EVALUATION OF A NITRATE ASSIMILATING BACTERIUM FOR POTENTIAL USE IN NITRATE BIOREMEDIATION

A Thesis
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

Deidra Angeline Lies

In Partial Fulfillment
for the Degree of
MASTER OF SCIENCE

Major Program:
Environmental and Conservation Sciences

May 2013

Fargo, North Dakota

North Dakota State University
Graduate School

Title

Evaluation of a Nitrate-Assimilating Bacterium for Potential Use in Nitrate Bioremediation

By

Deidra Angeline Lies

The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

SUPERVISORY COMMITTEE:

Dr. Peter Oduor
Chair

Dr. Penelope Gibbs

Dr. Frank Casey

Dr. Xinhua Jia

Approved:

June 24, 2013
Date

Dr. Craig Stockwell
Department Chair

ABSTRACT

Increasing levels of nitrate (NO_3-N) in water resources have brought about the need to find ever more versatile forms of NO_3-N removal from contaminated water. The ability of several microorganisms to transform NO_3-N has been embraced as an economical form of bioremediation. Free cell and immobilized forms of *Methylobacterium fujisawaense* ATCC® No. 35065™ (*M. fujisawaense*) were used in this study for NO_3-N removal in batch settings. NO_3-N removal results were as high as 100% for freely suspended cells after 96 hours and 95% for immobilized cells also after 96 hours. Statistical analyses found no significant difference in overall NO_3-N removal rates between free cell and immobilized systems. These findings suggest that the organism is capable of up to full assimilation of 10 mg/L NO_3-N in certain settings. The findings also suggest that the NO_3-N assimilating ability of *M. fujisawaense* of 10 mg/L NO_3-N is not greatly altered by immobilization.

ACKNOWLEDGMENTS

I humbly thank God for all the gifts I have received; for Him, nothing is impossible. I also need to thank my dear family and friends for all of their love, support, and encouragement throughout my entire graduate school experience; their patience and kindness were truly humbling and much appreciated. I would also like to extend many thanks to my academic advisor, Dr. Peter Oduor, for granting me the opportunity to pursue this research and my graduate studies. None of it would have been possible without his hard work and generosity. I would also like to express gratitude to my committee members, Dr. Penelope Gibbs, Dr. Frank Casey, and Dr. Xinhua Jia, for agreeing to provide me with their guidance and valuable insight. I also extend thanks to Dr. Donald Schwert for providing partial funding for this research.

I would also like to acknowledge and express my gratitude to Dr. Penelope Gibbs for her generosity with her time, knowledge, and laboratory equipment. I also am indebted to her laboratory's research specialist, Heather Vinson, for all the assistance and guidance so generously given me during my visits to Van Es Hall. I would also like to thank the dedicated consultants at the Statistical Consulting Service for their assistance. I also need to offer gratitude to my fellow ECS students and friends, especially Buddhika, Anthony, Seydou, Anar, Papia, Felix, Meridith, and Girish, for all of their support, encouragement, and assistance.

Many thanks also to the USDA, ND EPSCoR Program, NSF, and ND INBRE Program for funding this research study. The project described was supported by NIH Grant Number P20 RR016471 from the INBRE Program of the National Institute of General Medical Sciences.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX TABLES

LIST OF APPENDIX FIGURES

CHAPTER 1. INTRODUCTION

1.1. NO_3-N: occurrence in water

Reactive nitrogen is a term that encompasses the biologically available forms of the element; nitrogen as nitrogen gas is not considered reactive nitrogen since very few types of organisms, excluding nitrogen fixers, can use it as a nitrogen source (Galloway et al., 2003; Fields, 2004). Examples of reactive nitrogen include inorganic compounds, such as ammonia, nitric acid, and nitrate, and organic compounds, such as protein, nucleic acids, and urea (Galloway et al., 2003). Within the last century, the rate of reactive nitrogen entering the earth's ecosystems has doubled due to anthropogenic activities (Vitousek et al., 1997; Galloway et al., 2003). Major contributors to the increase have included the cultivation of nitrogen fixing crops and the production and use of industrial fertilizers (Vitousek et al., 1997; Galloway et al., 2003; Lambert and Driscoll, 2003). The total increase in reactive nitrogen world-wide has increased nitrate (NO_3-N) in the waters of the United States (Dubrovsky et al., 2010). A United States Geological Survey study that collected data from 1993 to 2003 found that 90% of the 190 streams sampled nation-wide had NO_3-N concentrations that exceeded their natural, expected levels (Dubrovsky et al., 2010). In addition, out of the 406 shallow, domestic wells sampled in agricultural areas, 20% exceeded the EPA's Maximum Contaminant Level (MCL) for NO_3-N in drinking water (Dubrovsky et al., 2010). Privately owned wells in agricultural areas are especially prone to NO_3-N contamination, likely due to the heavy use of agricultural fertilizers and their shallow depths (Ward et al., 1996). The aforementioned study (Dubrovsky et al., 2010) also projects an increase in NO_3-N concentration in deeper groundwater sources over the next decade as NO_3-N-laden shallow groundwater migrates downward. The natural process of denitrification, the microbial conversion of nitrate to nitrogen gas that occurs in groundwater, has

been shown to remove up to 1.2 mg NO_3-N/kg a day in natural aquifers (Korom, 1992). However, the amount naturally removed will depend on the availability of the necessary electron donors, for example organic carbons or reduced iron, and the presence of anaerobic conditions that allow microbial denitrification to take place. (Korom, 1992; Rivett et al., 2008; Korom et al., 2012). The study points out that with continued additions of NO_3-N to ground and surface water, NO_3-N levels of public water supplies that utilize deeper groundwater may also be affected (Dubrovsky et al., 2010).

1.2. NO_3-N: human health concerns

Human health concerns linked to NO_3-N in drinking water include occurrences of methemoglobinemia in infants, non-Hodgkin, gastric, bladder, and other cancers, and neural tube reproductive effects (Ward et al., 1996; Knobeloch et al., 2000; Ward et al., 2005). The connections between NO_3-N in drinking water and both various cancers and neural tube defects in fetuses have been evaluated in several studies, but a direct cause-effect relationship between these health concerns and NO_3-N in drinking water has not been definitively confirmed or disproven (Fan and Steinberg, 1996; Ward et al., 2005). Another human health concern with a connection to NO_3-N in water is that of infant methemoglobinemia, or Blue Baby Syndrome. Methemoglobinemia is a potentially fatal condition that occurs when nitrite oxidizes hemoglobin to methemoglobin, causing a reduction in the blood's ability to efficiently transport oxygen (Knobeloch et al., 2000; WHO, 2011). The connection between infant methemoglobinemia and high levels of NO_3-N in drinking water was first described in 1945 by Hunter Comly, who proposed that bacterial conversion of NO_3-N to nitrite could occur in an infant's gastrointestinal tract (Avery, 1999). As reported in Walton (1951), a national survey on infant methemoglobinemia by the American Public Health Association supported Comly's hypothesis

where reported cases of methemoglobinemia often occurred in tandem with elevated levels of NO_3-N in drinking water. The survey's results show that no instances of methemoglobinemia were found below 10 milligrams per liter of NO_3-N, the concentration set as the maximum contaminant level allowed in public water supplies by the Environmental Protection Agency in 1974 (Avery, 1999).

Some researchers (Avery, 1999; L'hirondel et al., 2006) have questioned the need for the maximum NO_3-N level to be as low as 10 mg/L NO_3-N due to the economic costs of maintaining such a low concentration in natural waters. In addition to cost, the picture of what causes infant methemoglobinemia, including the possibility of endogenous nitrate production during infections, has become more complex (Avery, 1999; L'hirondel et al., 2006). Proponents of increasing the allowed nitrate levels have also cited the scarcity of infant methemoglobinemia cases reported in the U.S. since the 1980s (Avery 1999). However, published reports of infant methemoglobinemia include two cases as recent as 1999 (Knobeloch et al., 2000). Some researchers have also pointed out that an unknown number of infant methemoglobinemia cases may go unreported because there are no reporting requirements in the U.S., and the less severe versions of the syndrome may be misdiagnosed (Knobeloch et al., 2000; Fewtrell, 2004). Some researchers have cautioned against any increase in the regulation before the complex relationship between NO_3-N in drinking water and possible adverse health effects is better understood (Knobeloch et al., 2000; Ward et al., 2006). Currently, the World Health Organization advises a maximum NO_3-N level of 11 mg/L NO_3-N for drinking water (WHO, 2011).

1.3. NO_3-N removal using biological processes: denitrification

Although physicochemical methods, ion exchange utilizing zeolites for example, are effective at the removal of NO_3-N and other soluble nitrogen forms from water, factors such as

their considerable cost and difficulties in maintenance, regeneration, and disposal have so far kept these processes from becoming dominantly used in the water treatment sector (Ergas and Reuss, 2001; Shrimali and Singh, 2001; Liu, 2007). In contrast, a more economical process, the biological process of denitrification by bacteria, has become a commonly used method for the removal of NO_3-N in both tertiary wastewater treatment and the bioremediation technologies used for treatment of groundwater (Metcalf and Eddy, 1991; Schipper and Vojvodic-Vukovic, 2000; EPA, 2007; Liu, 2007; Wang et al., 2009). Denitrification is a form of respiration performed by microbes capable of using the NO_3 ion as an electron acceptor under anaerobic conditions (Wrage et al., 2001). NO_3 is reduced sequentially to nitrite, nitric oxide, nitrous oxide, and finally to dinitrogen gas (Bothe et al., 2007). As effective and efficient as the denitrification route of NO_3 bioremediation is, the use of the denitrification process when oxygen conditions are not anoxic can lead to a shift in less dinitrogen production to more nitrous oxide production (Takaya et al., 2003). In wastewater treatment processes, for example, the aerobic phase of biological treatment required to reduce organic content and allow nitrification of ammonia to NO_3 to occur comes before the anoxic phase of denitrification. Water coming from the aerobic phase of treatment is not always brought to a completely anoxic level before its arrival at the denitrification phase (Takaya et al., 2003). Nitrous oxide production is a concern because the gas is a potent greenhouse gas, 200 to 300 times more potent than carbon dioxide in its contribution to global warming, and is also capable of ozone layer depletion (Munch and Velthof, 2007; Ravishankara et al., 2009). As outlined in Rowland (2005), nitrous oxide in the stratosphere serves as the major source of stratospheric nitrogen oxides (NO and NO2) upon its breakdown by electronically excited oxygen atoms. These electronically excited oxygen atoms are formed by the degradation of ozone by short wavelength ultraviolet light. The nitrogen

oxides formed serve as free radical catalysts in the degradation of ozone molecules to the more stable diatomic oxygen molecule, thus contributing to depletion of stratospheric ozone (Rowland, 2005). Ravishankara et al. (2009) reported that nitrous oxide from anthropogenic sources was the largest contributor to total ozone depleting emissions and was expected to remain the top contributor through the remainder of the 21st century.

1.4. NO_3-N removal using biological processes: assimilation

Nitrate assimilation by bacteria is another biological process that can remove NO_3-N from soil or water by transforming it into biomass; NO_3-N in the cell is reduced to nitrite, then the ammonium ion, and finally becomes integrated into organic forms of nitrogen needed for the cell (Metcalf and Eddy, 1991; Moreno-Vivian and Flores, 2007). In contrast to denitrification, the NO_3-N becomes part of the biomass, which is degradable by heterotrophic organisms, and so the nitrogen is considered reactive and available for use by living organisms (Galloway et al., 2003). In addition, NO_3-N assimilation does not carry the possibility of generating nitrous oxide under aerobic conditions because the NO_3-N is being immobilized into biomass and not respired (Bothe et al., 2007). However, the assimilation of NO_3-N in biomass would not serve as a permanent sink in an aqueous environment due to biodegradation (Korom, 1992). Thus, the biomass would need to be removed and recovered from the water to both prevent remineralization to NO_3-N and to take advantage of the nitrogen and other nutrients in the biomass, as is done with sludge from wastewater treatment plants (Metcalf and Eddy, 1991; Liu, 2007).

1.5. Advantages and issues of the use of polymeric carriers for living cells

One possibility for immobilizing NO_3-N assimilating bacteria in a bioremediation system is the use of polymeric carriers. Polymeric carriers are made using hydrogels, which are defined

as “three dimensional networks of polymeric material and water” that can surround and entrap macromolecules (Rosiak et al., 2002). Such carriers are made out of many different polymer-forming compounds and have seen success in studies in which they were used to immobilize microbes used for the bioremediation of NO_3-N contaminated water via denitrification (Hill and Khan, 2008; Siripattanakul et al., 2010; Song et al., 2005; Rezaee et al., 2008; Tenokuchi et al., 2006). The use of polymeric carriers to immobilize bacteria for use in bioremediation has been shown to have several practical benefits over a system with freely suspended cells. The prevention of cell washout from the system, easier product separation at the end of the process, a lower risk of contamination of system components, and higher levels of stability in biological processes are frequently mentioned immobilization benefits (Konsoula and Liakopoulou-Kyriakides, 2006; Pramanik and Khan, 2009).

Despite the many advantages of use of polymeric carriers in bioreactor settings, findings on whether or not their use affects the efficiency of the biological agents being immobilized are mixed. Pramanik and Khan (2008), for example, ran bioreactor experiments with mixed cultures of bacteria commonly used in wastewater treatment and found that the effect of immobilization on growth rate and substrate utilization rate when compared to a parallel free cell bioreactor was dependent on the source of the mixed bacterial culture being used. Konsoula and Liakopoulou-Kyriakides (2006) studied the effects of immobilization in calcium alginate beads on the cells *Bacillus subtilis* and their ability to produce the enzyme, α-amylase. They found that immobilization of the cells resulted in a 2.5 fold increase in enzyme production when compared to the free cell systems. In contrast, Pramanik and Khan (2009) found that with some pure cultures of wastewater treatment bacteria, the growth rate and substrate utilization rates of cells immobilized in calcium alginate were statistically lower or did not differ from the rates of their

free cell counterparts. The contrasting results of the studies suggest a range of possible effects of immobilization on a NO_3-N assimilating bacterium and its ability to uptake NO_3-N.

1.6. Research objectives

The NO_3-N removal capacity of one strain of *M. fujisawaense* and its response to immobilization was evaluated using the following objectives:

- Determine the degree to, and the rate of, which the NO_3-N assimilating bacterium, *Methylobacterium fujisawaense* (*M. fujisawaense*), can remove a NO_3-N level of 10 mg/L NO_3-N (EPA Maximum Contaminant Level).
- Explore the effect of immobilization on the NO_3-N assimilating ability of *M. fujisawaense* by immobilizing the bacterium on calcium alginate beads in a batch reactor and a flow through reactor, which were compared to the NO_3-N utilization rate of parallel free cell set-ups.

1.7. Hypotheses

Null: If immobilization has no significant effect on the nitrate-assimilating ability of the bacterium, then the overall nitrate removal trends in free cell and immobilized cell reactors will not vary significantly.

Alternative/Research Hypothesis: If immobilization does have a significant effect on the nitrate-assimilating ability of the bacterium, then the overall nitrate removal trends in free cell and immobilized cell reactors will vary significantly.

1.8. Rationale for use of calcium alginate beads as a polymeric carrier and *M. fujisawaense* as a potentially useful NO_3-N assimilating bacterium for bioremediation

Calcium alginate beads were chosen for use in this study because of their many favorable properties as polymeric carriers of biologically active agents. First, the beads exhibit physical

properties that would allow them to immobilize and retain active cells without negatively affecting their biochemical processes. The matrix formed from the calcium alginate is fairly inert and, by selecting the right concentrations of initial solutions, the level of porosity in the matrix will be high enough to allow sufficient diffusion rates of macromolecules necessary for the biological process of interest (Gombotz and Wee, 1998). Second, the preparation of such carriers is a simple process that can be performed with basic laboratory equipment at room temperature and without the use of harsh reagents. The amount of coating, and thus porosity, of the beads can be altered with ease by a change in concentration of the hardening solution used (Gombotz and Wee, 1998). These carriers can also be used in an actual bioremediation setting (Siripattanakul et al., 2010).

M. fujisawaense was acquired from the American Type Culture Collection (ATCC) in Manassas, VA. It is a biosafety level one organism, originally isolated from soil and water, with a recommended growth media that contains potassium nitrate (KNO_3) as the sole nitrogen source (ATCC, 2011). In addition to its suitable safety rating and ability to assimilate NO_3-N, the organism belongs to both a genus and species whose members have shown various forms of bioremediation potential in past studies (Section 2.1 of Literature Review).

1.9. Organization of thesis

This thesis describes the results of experiments performed to develop an initial evaluation of the NO_3-N removal capability of *M. fujisawaense* when both freely suspended and immobilized on calcium alginate beads. The thesis begins with an introduction in Chapter 1, followed by a literature review in Chapter 2. Chapter 3 describes the methodology of the experiments performed, followed by the experimental results in Chapter 4 and a discussion of the

results in Chapter 5. Chapter 6 provides a conclusion to the thesis. References and appendices are found at the end of the thesis.

CHAPTER 2. LITERATURE REVIEW

2.1. Nitrate in the nitrogen cycle: microbial processes and bioremediation

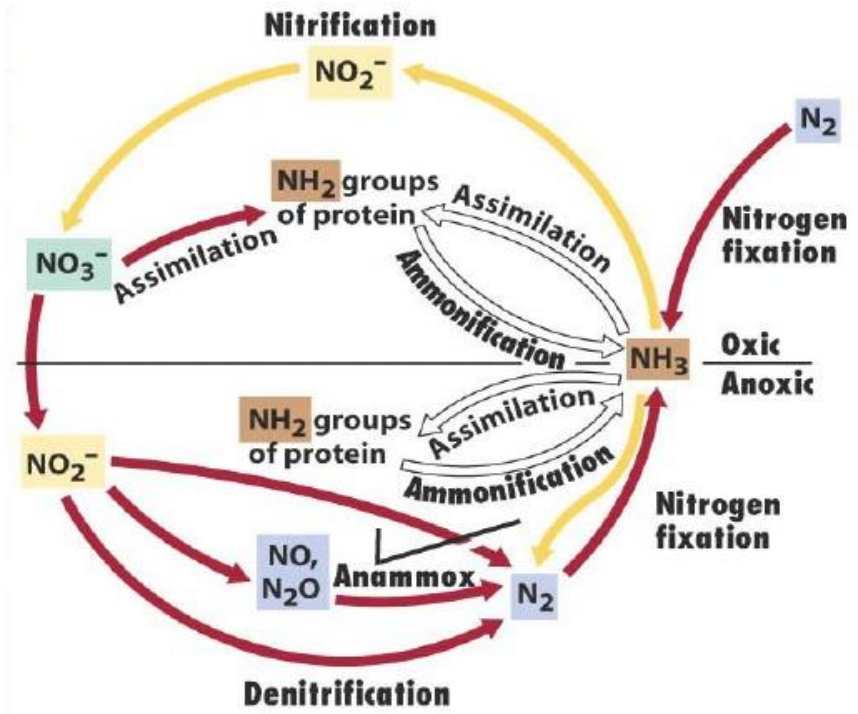


Figure 2.1. Nitrogen redox cycle: red arrows = reductions, yellow arrows = oxidations, white = reactions with no redox change (Madigan et al., 2005).

Nitrate, the most oxidized state of nitrogen, can be reduced via three separate microbe-mediated processes. Dissimilative reduction of nitrate to ammonia (the horizontal oxic-anoxic black line), can be the prevailing nitrate reducing process in anoxic settings where there is an abundance of electron donors such as organic matter (Madigan, et al., 2012). In terms of nitrate bioremediation from a water source, the process is less than ideal because it transforms nitrate to ammonia, another possible contaminant. Upon arrival to an aerobic environment in the water source, the ammonia would be oxidized back to nitrate via the nitrification pathway (Rivett et al., 2008).

The second nitrate-reducing pathway, nitrate assimilation, occurs when certain heterotrophic microbes take up and reduce nitrate to organic nitrogen compounds. These microbes, such as *M. fujisawaense*, use nitrate as their nitrogen source for biomass synthesis (Rivett et al., 2008). This process can occur under oxic or anoxic conditions, depending upon the microbes involved (Kobayashi and Ishimoto, 1973; Ulehlova, 1988) and can be a significant nitrate-removal pathway in soil and groundwater under certain conditions (Kelso et al., 1999; Bengtson and Bengtsson, 2005; Rivett et al., 2008). The process requires the availability of organic electron donors and, in a bioremediation setting, the ability to remove the resulting microbial biomass. If the biomass remains in the water source, the microbes will eventually die and return the nitrogen to the water as ammonium (Rivett et al., 2008).

Denitrification is the third nitrate-reducing microbial pathway. Denitrifying microbes use nitrate as an electron acceptor in their respiratory processes and emit the resulting nitrogen gas (N_2) or nitrous oxide (N_2O) (Korom, 1992). Depending on the denitrifying microbe, either an organic or inorganic electron donor will be necessary for denitrification to occur. The majority of denitrifying microbes are facultative anaerobes that perform denitrification once the dissolved oxygen concentration in the environment drops below 1 to 2 mg/L (Rivett et al., 2008). Denitrification is the dominant nitrate attenuating process in anoxic groundwater settings. It physically removes nitrate from the water source via the release of nitrogen gas (Korom, 1992; Rivett et al., 2008; Korom et al., 2012). It has seen success as a form of nitrate bioremediation (Metcalf and Eddy, 1991; Schipper and Vojvodic-Vukovic, 2000; EPA, 2007; Liu, 2007; Wang et al., 2009), but it cannot be performed efficiently under aerobic conditions (Korom, 1992; Rivett et al., 2008). An increase in nitrous oxide formation may also occur if oxygen or nitrate levels are increased in the system (Takaya et al., 2003; Rivett et al., 2008).

2.2. Profile of a NO_3-N-assimilating bacterium with possible bioremediation potential

Occurrences of NO_3-N and other chemicals as co-contaminants in water systems are also examples of settings where NO_3-N immobilizing bacteria with capacity for tolerance, or the ability to biodegrade, the co-contaminant may become useful for bioremediation purposes. For example, Vasiliadou et al. (2008) used a mixed culture of bacteria isolated from olive pulp to both biodegrade phenol, a common groundwater contaminant, and assimilate NO_3-N. The mixed culture was able to continue to biodegrade and assimilate nitrate in the presence of concentrations up to 950 mg/L phenol and 100 mg/L NO_3-N in aerobic batch reactors.

One possible candidate for such use is a member of the *Methylobacterium* genus of bacteria. *Methylobacterium fujisawaense* is a member of the α-2 subclass of the large class of *Proteobacteria* in the order, *Rhizobiales*, family, *Methylobacteriaceae*, and the genus, *Methylobacterium* (Garrity et al., 2005). *Methylobacterium fujisawaense* and fellow members of the genus, *Methylobacterium*, are strictly aerobic and often included in the group of pink-pigmented facultatively methylotrophic bacteria due to their common pink or red coloring from carotenoids and the genus's characteristic ability to use various single carbon compounds as carbon and energy sources (Urakami et al., 1993; Hiraishi et al., 1995). Members of the genus are also a) chemoorganotrophic, b) mesophilic (25-30 °C), c) gram-negative, d) motile via flagellum, e) mostly found singly, and f) rod-shaped with a width of 0.8 to 1.2 micrometers and a length ranging from 1.0 to 8 micrometers (Urakami et al., 1993; Garrity et al., 2005). Many members of the genus are capable of using compounds with multiple carbon bonds as a carbon source since they are facultative in their methylotrophy (Patt et al., 1976). Common nitrogen sources for the genus include NO_3, ammonia, and urea (Garrity et al., 2005). Although dependent on the species, many genus members can be grown on glycerol peptone agar and

methanol mineral salts medium (Garrity et al., 2005). Species of the genus are widespread in the environment and can be found in soil, air, freshwater, sediment, the phyllosphere, and the rhizosphere. (Hiraishi et al., 1995; Van Aken et al., 2004a; Madhaiyan et al., 2007). The pink-pigmented facultatively methylotrophic bacteria can also endure extreme environmental conditions of dryness, freezing, radiation, and high temperatures (Trotsenko et al., 2001).

As mentioned, some species are commonly found in association with plants (Van Aken et al., 2004a; Madhaiyan et al., 2009; Schauer et al., 2011). One study found that the pink-pigmented facultatively methylotrophic bacteria, including *Methylobacterium*, make up an average of 36% of the total microbial heterotroph count on young leaf surfaces (Corpe and Rheem, 1989). Many of these plant dwelling species use the methanol produced during plant metabolism as both a carbon and energy source (Trotsenko et al., 2001). Some species can also be considered symbionts as they have been shown to stimulate growth or survival in some plant species via production of plant growth-inducing hormones and vitamins (Trotsenko et al., 2001; Kutschera and Koopmann, 2005). For example, other strains of *Methylobacterium fujisawaense* have been found to increase cytokinin concentrations and the root elongation of canola plants (Madhaiyan et al., 2006). In addition, many methylotrophic groups of bacteria have been found to be capable of the production of the exopolysaccharides needed to form biofilms (Schrader et al., 2008; Rosseto et al., 2011). This ability would allow the plant dwelling species of pink-pigmented facultatively methylotrophic bacteria to persist on the surface of plants and endure harsher environmental conditions (Trotsenko et al., 2001).

Several members of *Methylobacterium* have also been found to be capable of enduring and biodegrading certain toxic compounds. For example, several species in the genus were found to be capable of biodegradation of the toxic explosives 2,4,6-trinitrotoluene, hexahydro-1,3,5

trinitro-1,3,5-triazine, and octahydro-1,3,5,7-tetranitro-1,3,5-tetrazocine (Van Aken et al., 2004b). Mo et al. (1997) discovered that a pure culture of *Methylobacterium mesophilicum* ATCC® No. 700107™ was capable of biodegrading 200 ppm of methyl tert-butyl ether, a common gasoline oxygenate and groundwater contaminant, by 29% after two weeks of incubation. Idris et al. (2006) found that three species of *Methylobacterium* living in association with the nickel hyper-accumulating plant, *Thlaspi goesingense*, were tolerant to the metals nickel, cadmium, cobalt, zinc, and chromium. In De Marco et al. (2004), of the 31 contaminant tolerant methylotrophs isolated from soil and sediment in the study, 11 came from *Methylobacterium* and three of the top four most widely tolerant strains were members of *Methylobacterium*. One of the top three most tolerant was a strain of *Methylobacterium fujisawaense* that had been isolated from acidic forest soil. It could tolerate the organics methyl tert-butyl ether, naphthalene, xylene, styrene, benzene, phenol, crude oil, and the heavy metals arsenate, cadmium, chromate, mercury, and lead. The strain could also tolerate temperatures from 8 to 37 °C and pH levels from 5 to 12 (De Marco et al., 2004). In a study of eight bacterial isolates from water treatment and distribution systems, Zhang et al. (2009) found that the strain *Methylobacterium fujisawaense* PAWDI had the second highest degradation rate of dichloroacetic acid, an EPA regulated disinfection byproduct in treated drinking water.

2.3. Use of polymeric carriers in NO_3-N bioremediation

Researchers in the field of NO_3-N bioremediation have studied the ability of various polymeric materials to serve as carriers for denitrifying bacteria. The use of such polymeric materials to immobilize microbes imparts several benefits to an aqueous system when compared to such a system operating with freely suspended microbes. Benefits include the increased concentration and retention of microbial cells within the system and less need for solids removal

after the denitrification process has occurred (Hill and Khan, 2008). Some immobilization techniques have been shown to reduce NO_3-N concentrations more effectively when directly compared to their free cell system counterparts (Siripattanakul et al., 2010).

The practice of immobilization has been combined in its various forms with the equally numerous species of microbes capable of transforming NO_3-N via denitrification (Breisha and Winter, 2010). Heterotrophic denitrification is a common form of NO_3-N utilization in many microbes and is also a common pathway utilized for NO_3-N removal from contaminated water (Breisha and Winter, 2010). The process requires the addition of a carbon source to fuel microbial reduction of NO_3-N and can be performed by either a single species or a mixed culture of microbes (Breisha and Winter, 2010).

Siripattanakul et al. (2010) tested the ability of a mixed culture of heterotrophic, denitrifying microbes to remove NO_3-N from simulated agricultural wastewater both when immobilized on calcium alginate beads and as freely suspended cells. The researchers passed the simulated agricultural wastewater containing NO_3-N, methanol, and free or immobilized bacteria through sterile sand columns and measured the NO_3-N and nitrite concentrations in the effluent exiting the columns. Using an initial NO_3-N concentration of 50 milligrams per liter in the solution and methanol as a carbon source, they observed after eight hours: a 90% to 99% reduction of NO_3-N when using immobilized microbes and a 56% to 75% reduction with freely suspended microbes. The authors observed no accumulation of nitrite in either the immobilized or free cell system after eight hours (Siripattanakul et al., 2010).

Other researchers have tested immobilization methods using a single species of denitrifying microbe. For example, Song et al. (2005) used a novel combination of polyvinyl alcohol, xanthan gum, and Tween 20 surfactant to create capsules to immobilize the denitrifier

Ochrobactrum anthropi SY509. This combination was meant to create a carrier with more stability and cell density capacity than single or simple combinations of polymeric materials used to immobilize microbes. Using glucose as a carbon source, the denitrifying bacteria immobilized on these new capsules achieved a reduction of 100 mg/L NO_3-N to zero in 45 minutes; in comparison, cells immobilized on polyvinyl and calcium alginate capsules required two hours for the complete removal of the same concentration of NO_3-N, while cells immobilized on polyvinyl alcohol capsules only lowered the concentration by 50% to 50 mg/L of NO_3-N (Song et al., 2005). Rezaee et al. (2008) also used a single heterotrophic bacterium for their continuous flow denitrification study. They utilized "microbial cellulose", a natural polysaccharide mesh produced by *Acetobacter xylinum*, as the immobilization material for the denitrifying microbe *Pseudomonas stutzeri* (Rezaee et al., 2008). Using an influent with an initial NO_3-N concentration of 200 mg/L, NO_3-N levels were lowered from the initial concentration by as much as 99% over a 20-day operation period. Effluent levels also did not rise above 7.53 mg/L NO_3-N at any point throughout the 20 days (Rezaee et al., 2008).

Although experiments with both heterotrophic denitrification and polymeric carriers are more numerous, immobilization experiments using the process of autotrophic denitrification are being conducted as well (Breisha and Winter, 2010). Autotrophic denitrification processes take advantage of the ability of autotrophic microbes to use carbon dioxide as a carbon source; this ability eliminates the need for an organic carbon input in the denitrification process (Breisha and Winter, 2010). Tenokuchi et al. (2006) immobilized the denitrifying bacteria *Paracoccus denitrificans* IFO13301 on a new form of "microcapsules" formed from sodium alginate solution, dichloromethane, and polymethylmethacrylate. The researchers tested the NO_3-N removal efficiency of the encapsulated bacteria via autotrophic denitrification using the

following: 100 mL batch reactor systems supplied with 20 mg/L NO_3-N and hydrogen gas as an energy source. They found that the inoculated capsules could completely remove the 20 mg/L NO_3-N and any resulting nitrite from the reactors; the complete removal of both nitrogen oxides required 18 to 50 hours. The inoculated capsules could also be used to reduce NO_3-N concentrations in batch reactors four times before needing re-incubation (Tenokuchi et al., 2006).

Other researchers have used novel adaptations of immobilized denitrifying microbes to perform NO_3-N bioremediation. Kim et al. (2007) used cells of the denitrifier *Ochrobactrum anthropi* SY509 in an "electrochemical bioreactor" to decrease NO_3-N concentrations without the use of a carbon source. They immobilized the permeabilized, thus dead, bacterial cells in a sodium alginate solution that was spread on a graphite electrode; the denitrifying enzymes within the cells received the electrons needed to carry out NO_3-N reduction from the electrode. Starting at an initial concentration of 140 mg/L NO_3-N, and with the use of an electrode in lieu of a carbon source, the system was able to reduce the initial NO_3-N concentration to 55 mg/L, with no accumulation of nitrite, in 3 hours.

CHAPTER 3. METHODOLOGY

3.1. Growth curve construction for *M. fujisawaense*

A growth curve describes a full cycle of growth for a population of organisms growing in a closed, or batch, system. The curve can be divided into the lag, exponential, stationary and death phases of growth for the population of organisms being studied (Madigan et al., 2012). In order to have a timeline for growth rate for the strain, *M. fujisawaense*, and to have spectrophotometer readings of optical density at 600 nm ($OD_{600)}$ to pair with average colony forming unit counts per mL (CFUs/mL), growth curve construction for the organism was undertaken. Broth medium ATCC® 1354 was prepared and contained the following per liter of deionized water: 1 g of $MgSO_4$, 0.2 g $CaCl_2$, 2 mL chelated iron solution, 1 g KNO_3 , 0.5 mL trace element solution, 0.272 g KH_2PO_4 , and 0.717 g Na_2HPO_4. The chelated iron solution contained the following per 100 mL of deionized water: 0.1 g ferric (III) ammonium citrate, 0.2 g EDTA, sodium salt and 0.3 mL concentrated HCL. The trace element solution contained the following per one liter of deionized water: 500 mg EDTA, 200 mg $FeSO_4 \cdot 7H_2O$, 10 mg $ZnSO_4 \cdot 7H_2O$, 3 mg $MnCl_2 \cdot 4H_2O$, 30 mg H_3BO_3, 20 mg $CoCl_2 \cdot 6H_2O$, 1 mg $CaCl_2 \cdot 2H_2O$, 2 mg $NiCl_2 \cdot 6H_2O$, and 3 mg $Na_2MoO_4 \cdot 2H_2O$. The medium was autoclaved at 121 °C for 15 minutes and one mL of filter-sterilized methanol was added after as the carbon source. 30 mL of ATCC® 1354 broth media was inoculated with one colony of *M. fujisawaense* and incubated on an 18 rpm rocker at 25 °C. Spectrophotometer readings (600 nm) on a Thermo-Scientific Biomate 3™ and dilution series plates were then taken and made three times daily until a consistent decline or stagnation in spectrophotometer values occurred. Two more growth curves were generated for *M. fujisawaense,* but the 80 mL cultures were agitated at 200 rpm to ensure non-limiting oxygen levels and incubated at an average temperature of 28 °C. Spectrophotometer readings and

dilution plates were also taken and made using the same schedule as the first growth curve. Growth curves were generated by graphing time against spectrophotometer readings (OD_{600}) and time against colony forming units per mL broth culture (CFUs/mL).

3.2. Test of NO_3-N utilization by free bacterial cells – quantity and minimum growth requirements

In order to determine the rate and magnitude of NO_3-N utilization by *M. fujisawaense* as a freely suspended culture and to further define its growth requirements, batch experiments with duplicates of each treatment were conducted in a laboratory setting. Since most bacterial NO_3-N and nitrite reductase assimilation enzymes have been found to contain molybdenum and iron, (e.g. Lin and Stewart, 1998 & Moreno-Vivian and Flores, 2007) a test to affirm the necessity of inclusion of the bacterium's trace element solution containing these elements was conducted. A stock solution with a stoichiometric NO_3-N concentration of approximately 45 mg/L NO_3 (10 mg/l NO_3-N) was prepared by dissolving 0.07337 g of KNO_3 in one liter of deionized water. The solution was then autoclaved at 121 °C for 15 minutes. Thirty mL volumes were then aseptically transferred into eight sterilized 50 mL Kimax™ culture tubes. Two tubes containing 30 mL of NO_3-N solution and no further additions along with two tubes containing the NO_3-N solution plus 30 µL of filter sterilized methanol and 15 µL of 10x sterile (ATCC 1354) trace element solution served as controls. Two tubes had the addition of 30 µL of filter sterilized methanol and were then inoculated with 1 mL of log phase *M. fujisawaense* broth culture. The final two tubes received the additions of 30 µL of filter sterilized methanol, 15 µL of sterile 10x trace element solution and a 1 mL inoculation of the same log phase *M. fujisawaense* culture. The quantities of methanol and trace element solution added were chosen to result in 1 g/L and 5 g/L concentrations, respectively. This methanol concentration was equal to that found in the growth

medium of the organism while the trace element concentration was ten times the concentration of the growth medium. These levels were used to ensure that NO_3-N would be the limiting nutrient in the experiment. The tubes were then placed on 18 rpm rockers and incubated at 25 °C. Three, 1 mL samples were removed daily from each tube, filtered using a 0.22 μm polysulfone syringe filter (Aladn™ brand), and diluted in deionized water by a factor of ten in order to measure the NO_3-N concentration via ion exchange chromatography in a Dionex ICS-2000 system. Samples of 100 μL of the *M. fujisawaense* containing tubes were taken at each sample time to make dilution series plates and establish a measure of cell concentration throughout the experiment.

3.3. Comparison of NO_3-N utilization between free and immobilized cells

To compare the NO_3-N utilization between freely suspended and immobilized *M. fujisawaense* cells, batch experiments in duplicate were performed in a laboratory setting. Simulated NO_3-N-contaminated water was prepared and sterilized as previously described with resulting concentrations of 1 g/L methanol, 5 g/L trace element solution, and 10 mg/L NO_3-N. Bacterial cells for both the free cell and immobilized cell treatments were grown in ATCC® 1354 liquid growth media for 72 hours before being harvested via centrifugation at 4,000 rpm for 25 minutes in a Hettich Rotina 35 R centrifuge. The calcium alginate bead method used followed the methods of Konsoula and Liakopoulou-Kyriakides (2006) with the modification of Pramanik and Khan (2009). The modification consisted of hardening the beads overnight in the calcium chloride solution, as well as the use of 2% w/v calcium chloride solution in place of the 3.5% w/v calcium chloride solution of Konsoula and Liakopoulou-Kyriakides (2006). After hardening overnight in 2% calcium chloride solution, the beads inoculated with 0.2% (ww/v) *M. fujisawaense* were rinsed with sterile, deionized water. Five mL of the inoculated beads were

Table 3.1. Experimental matrix for NO_3-N utilization experiment.

Description	Control/Treatment	Volume (mL)	Temperature (°C)	NO_3-N(mg/L)	Methanol (g/L)	Trace Elements (g/L)
NO_3-N	Control	30	25	approx 10	0	0
NO_3-N/Methanol/ Trace Elements	Control	30	25	approx 10	1	5
NO_3-N/Methanol/ Bacteria	Treatment	30	25	approx 10	1	0
NO_3-N/Methanol/ Trace Elements/Bacteria	Treatment	30	25	approx 10	1	5

then placed in 20 mL of the simulated NO_3-N-contaminated water in sterile, 50 mL Kimax™ culture tubes. For the free cell treatment, the same NO_3-N solution was inoculated with an equal mass of *M. fujisawaense* as the beads (30 mg), and 20 mL of the inoculated solution were placed in sterile 50 mL Kimax™ culture tubes. Both treatments were run in duplicate. To serve as a control, 5 mL of blank beads were placed in 20 mL of the NO_3-N solution, also in 50 mL sterilized Kimax™ culture tubes. The blank preparation was also run in duplicate. All treatment and control tubes were placed on 18 rpm tube rockers and were incubated at 25 °C. One mL samples were removed daily from each tube and centrifuged at 18,000 rpm for 15 minutes in order to remove the suspended bacteria. The resulting supernatant was diluted in deionized water by a factor of ten in order to measure the NO_3-N concentration via the previously mentioned Dionex ICS-2000 system. One hundred μL aliquots of the inoculated treatment tubes *M. fujisawaense* were taken at each sample time to make dilution series plates to establish both a measure of cell concentration in the free cell treatment and a measure of cell release from beads in the immobilized treatment.

3.4. Comparison of NO_3-N utilization in free and immobilized cells with aeration

A larger volume version of the previously described experiment was also performed with certain variations in order to ascertain if the use of a tube rocker was causing oxygen-limiting conditions for the aerobic cells. To remedy any oxygen limiting conditions, air stones connected to Tetra™ brand air pumps were used for aeration in the experimental setup instead of the tube rockers. The larger volume experiment was also undertaken to give a larger yield of potential sample volume, allowing for the analysis of NO_3-N, pH, temperature, and to simulate a bench-scale aerated batch reactor as found in other studies (e.g. Hill and Khan (2008); Song et al. (2005); Rezaee et al. (2008); and Tenokuchi et al. (2006)). To grow the bacterial cells, two 250

mL Erlenmeyer flasks were filled with 100 mL each of ATCC® 1354 liquid media and inoculated with *M. fujisawaense*. The flasks were shaken at 200 rpm and 25 °C for 72 hours to allow for sufficient cell concentration and to attain cells in their log phase of growth. The cells were then harvested by centrifuging 1 mL volumes of the culture in microcentrifuge tubes at 20,800 rcf for 25 minutes. Five hundred milligrams (wet weight) of the resulting bacterial pellets were added to 250 mL of 2% alginic acid solution, and beads were made according to the previously described method of Konsoula and Liakopoulou-Kyriakides (2006) with the modification of Pramanik and Khan (2009). In place of hand-syringing, a VWR™ peristaltic micro-pump was used to add the 2% alginic acid solution drop-wise to the 2% $CaCl_2$ solution, allowing for greater consistency in size of the beads (average diameter 3 mm). The beads were then allowed to harden overnight in the 2% $CaCl_2$ solution. The $CaCl_2$ solution was then drained from the beads, and the beads were rinsed with deionized water. A volume of 750 mL of the sterilized simulated NO_3-N solution with 10 mg/L NO_3-N, 1 g/L methanol, and 5 g/L trace element solution was then added to the beads to give a total volume of one liter. A volume of 750 mL of the same solution was also inoculated with 500 mg (wet weight) of the bacterial pellets in a separate reactor to serve as a free cell comparison to the immobilized cells in the first reactor. Glass bottle reactors were aerated constantly through the air stones (Figure 3.1), and samples were taken from each reactor daily for NO_3-N, temperature, and pH analysis. NO_3-N analysis was done by filtering the samples taken with 0.22 μm filter syringes (Aladn™ brand) and using the Dionex ICS-2000 system. Bacterial growth in the free cell reactors were measured by spectrophotometer readings (600 nm) of cell concentration in the final two runs and by dilution series plating of cell counts in the free cell reactor in the final run (Madigan et al., 2012). Dilution series plating was performed to account for spectrophotometer readings that extended

beyond the range of the original growth curve generated. The immobilized and free cell reactors were run in triplicate trials and bead controls were run in duplicate trials for NO_3-N analysis.

Figure 3.1. Photograph of batch experiment reactors.

3.5. NO_3-N utilization in a flow through filter experiment

A bench-scale trickle filter experiment was used to evaluate the level of NO_3-N utilization by *M. fujisawaense* in a flow through setting. A volume of 250 mL of inoculated calcium alginate beads were made as previously described in order to parallel the amount of beads and bacteria used in the batch reactor experiment. The beads were placed in a three chamber filter reactor (Figure 3.2.), and the bottom opening of each chamber was lined with Whatman™ (Cat No. 1002 110) qualitative filter paper to prevent bead escape. One liter of the simulated NO_3-N solution with 10 mg/L NO_3-N, 1 g/L methanol, and 5 g/L trace element solution was delivered to the top of the filter apparatus via a Scientific Systems Inc. Mighty-Mini™ piston pump at the rate of 0.1 mL per minute (3,447 kPa). Aeration was provided by a Tetra™ 20 gallon aquarium air pump via an inlet adjacent to the solution delivery inlet at the top of the filter apparatus. Samples were collected daily from the bottom port of the filter set-up for

all three trials and pH and temperature were measured at the time of collection for the final two trials. NO_3-N analysis was conducted using the Dionex ICS – 2000 system.

Figure 3.2. Photographs of flow through filter, top (left) and profile (right).

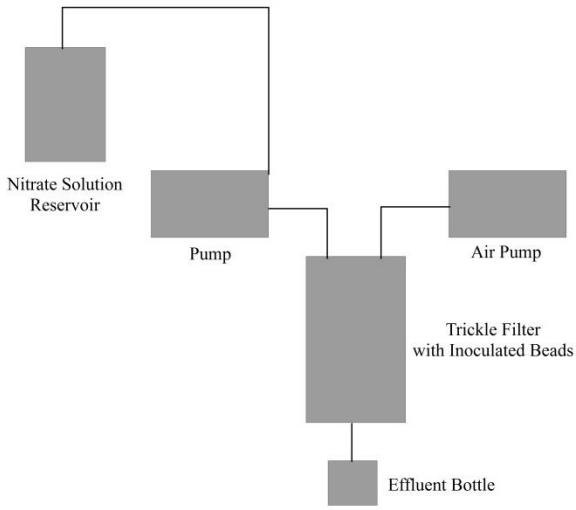


Figure 3.3. Schematic diagram of flow through filter experiment.

CHAPTER 4. RESULTS

4.1. Growth curve construction for *M. fujisawaense*

Average CFUs/mL counts showed a consistent upward trend (Figure 4.1) until hour 117, giving an exponential phase mid-point of 58.5 hours. Song et al. (2005) harvested cells for immobilization during the late exponential phase of growth, and the ATCC recommendation for use of M. fujisawaense is after 48 to 72 hours of incubation (ATCC, 2011). Therefore, an incubation period of 72 hours was chosen for the cultures used in experiments to capture the cells in an active, healthy state of growth. This followed both the ATCC recommendation and the procedure used by Song et al. (2005).

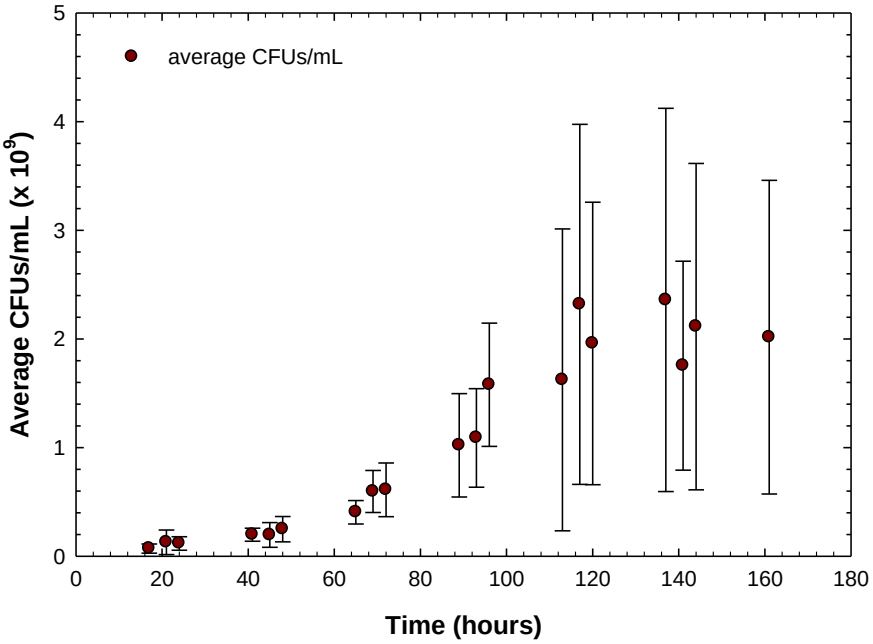


Figure 4.1. Growth curve results – average CFUs/mL.

4.2. Test of NO_3-N utilization by free bacterial cells – quantity and initial evaluation of minimum growth requirements

NO_3-N utilization tests with controls and bacterium-inoculated treatments were conducted.to validate that the growth of *M. fujisawaense* did bring about a significant reduction in levels close to 10 mg/L NO3-N. The same experiments were also used to ascertain if growth

could occur on a more minimalized growth media containing only carbon and nitrogen sources with the media-prescribed trace elements. Results from the initial comparison of controls with inoculated treatments are shown below in Figure 4.3.

The daily nitrate concentrations of the two controls were analyzed using the analysis of variance (ANOVA) and Tukey Methods with Minitab® 16 software at a 5% significance level. Average daily nitrate concentrations were not statistically different within control one or control two throughout the sampling period. Although 24 hour sampling was not performed, the control concentrations throughout the sampled time period varied little from the average concentration of 7.8 mg/L NO_3-N found in the initial stock NO_3-N solution used for the controls. The 7.8 mg/L NO_3-N found in the stock solution possibly differed from the stoichiometric value of 10 mg/L NO_3-N calculated for the stock solution due to minute spillage when working with a small amount of potassium NO_3-N reagent (less than 0.1 g). The largest difference in average concentration in mg/L NO_3-N between any two time periods measured was 0.3 and 0.4 for the NO_3-N only control and the NO_3-N, methanol and trace elements control, respectively. As depicted in Figure 4.3, the treatment inoculated with M. fujisawaense and with trace elements (treatment two) added showed reduction throughout the measured 48 to 168 hour time period. NO3-N concentration averages for the treatment containing only the bacteria and its nitrogen and carbon sources (treatment one) show no real decline and were actually higher than the NO_3-N stock solution concentration of 7.8 mg/L NO_3-N. Averages greater than the average initial NO_3-N solution concentration of 7.8 mg/L NO_3-N indicated an unmeasured addition of NO_3-N from the mL of bacterial culture used to inoculate the treatments. Although this makes the initial NO_3-N concentration for the treatments unknown, it can be surmised that both started at similar elevated concentrations since both were inoculated with the same bacterial culture that had been

mixed continuously to allow for aeration. Averages for NO3-N concentrations in the treatment containing bacteria, its carbon and nitrogen sources, and trace elements (treatment two) show consistent decline throughout the whole time period, with the lowest average concentration of 0.8 mg/L NO_3-N occurring after 168 hours. The initial NO_3-N concentration for the treatments was not able to be calculated due to the unmeasured addition from the inoculate and was therefore higher than the average stock concentration of 7.8 mg/L NO_3-N. A conservative minimum of 89.7% NO_3-N reduction can be reported to have occurred in treatment two, using the underestimated starting concentration of 7.8 mg/L NO3-N. This comparison reinforced the concept of microbial growth requiring trace elements, and they were used throughout the experiments conducted.

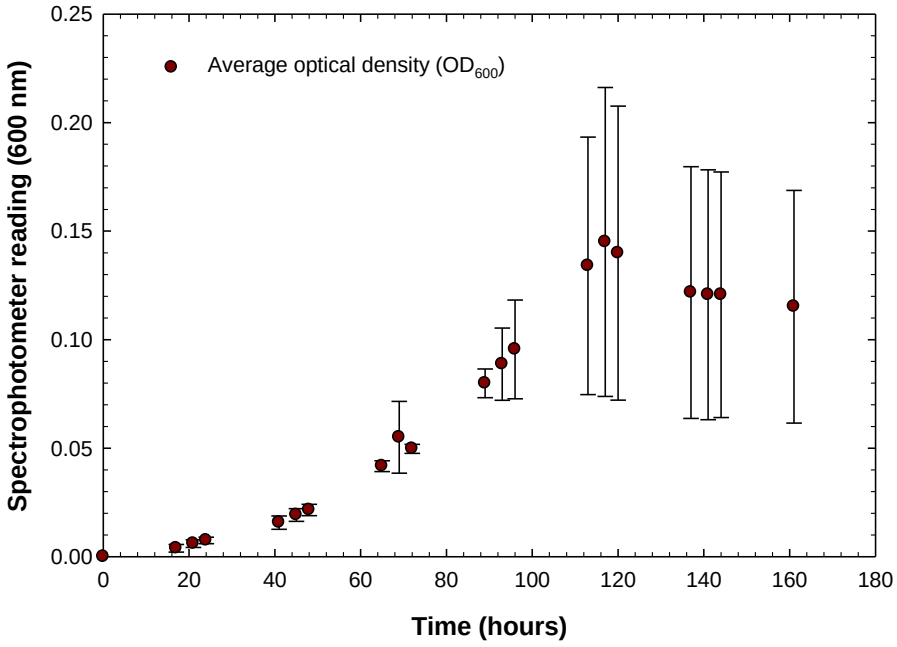


Figure 4.2. Growth curve results – average OD_{600}.

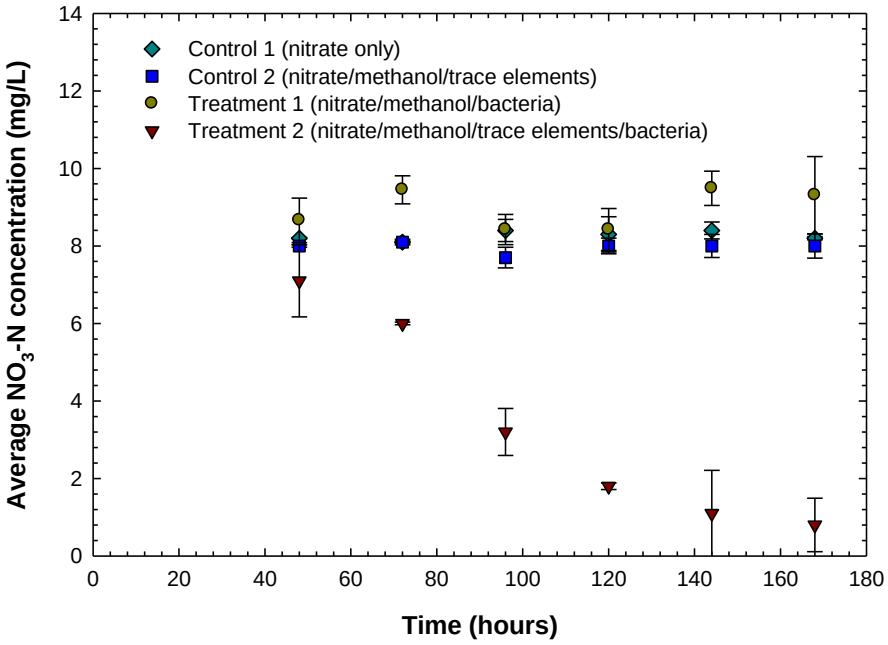


Figure 4.3. Results of NO_3-N assimilation in NO_3-N utilization experiment.

Figure 4.4 shows the average CFUs/mL per time period for the treatments (1 - no trace elements and 2 - with trace elements) from the NO_3-N utilization experiment. Treatment two shows an increase in CFUs/mL until after 144 hours at which point the numbers of colonies peak at an average of about 2.7×10^9 per mL and then began to level off and decline. Treatment one shows growth, but the counts of CFUs/mL measured from hours 72 to 168 are smaller than those of treatment two by a factor of ten or more. The highest average colony account achieved for treatment one was 7.6×10^7 per mL after 144 hours. Since both treatments were inoculated with the same bacterial culture, it was assumed that both treatments started at a similar concentration of both NO_3-N and bacterial cells.

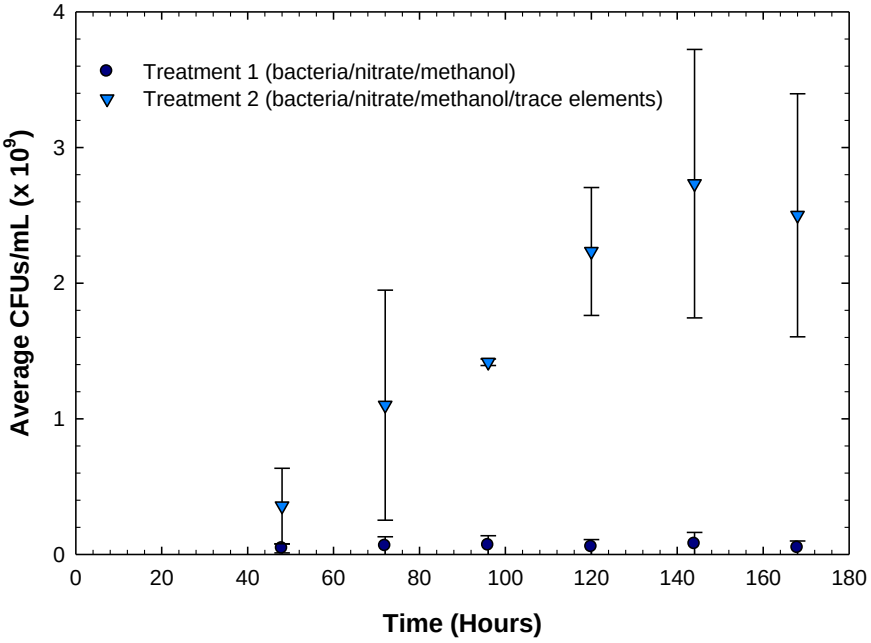


Figure 4.4. Bacterial growth in CFUs/mL for NO_3-N utilization experiment.

4.3. Comparison of NO_3-N utilization in free and immobilized cells

After determining that *M. fujisawaense* was capable of at least 89.7% NO_3-N removal of approximately 10 mg/L NO_3-N with both a carbon source of methanol and trace elements, an experiment was conducted to test the effect of immobilization on the NO_3-N assimilation ability of the bacterium. Resulting NO_3-N concentrations are shown below in Figure 4.5 for control beads, inoculated beads, and free cells.

An increase in NO_3-N concentration occurred in all three settings after 144 hours. The concentration in the free cell treatment increased from 0.05 mg/L NO_3-N after 120 hours back to 5.9 mg/L NO_3-N after 144 hours, while the bead treatment concentration increased from 0.5 to 4.5 mg/L NO_3-N during the same time period. This coincided with a decrease in cell count of approximately 45% for both treatments in between hour 120 and 144 (shown in Figure 4.6 below).

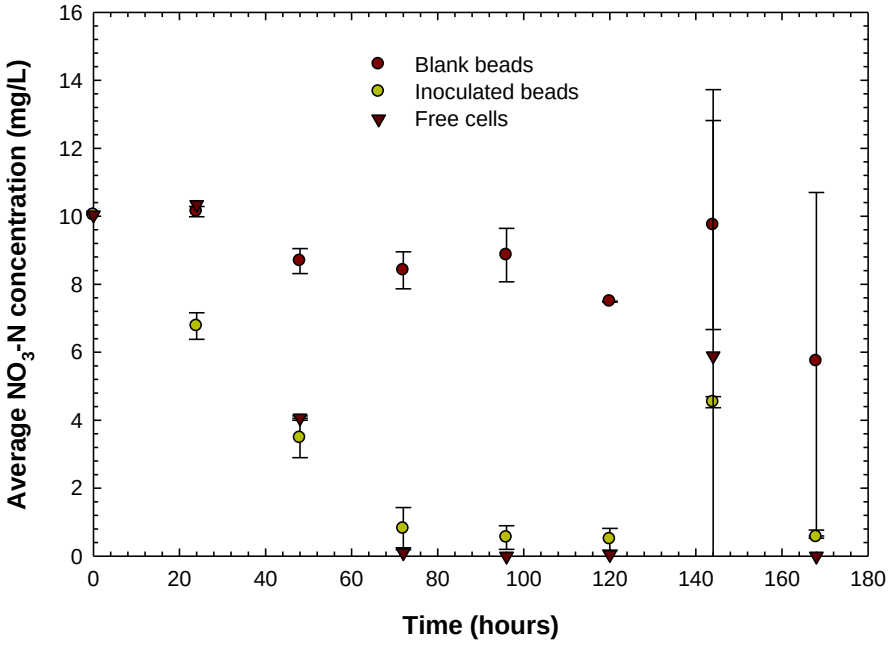


Figure 4.5. NO_3-N assimilation results for control beads, inoculated beads, and free cells.

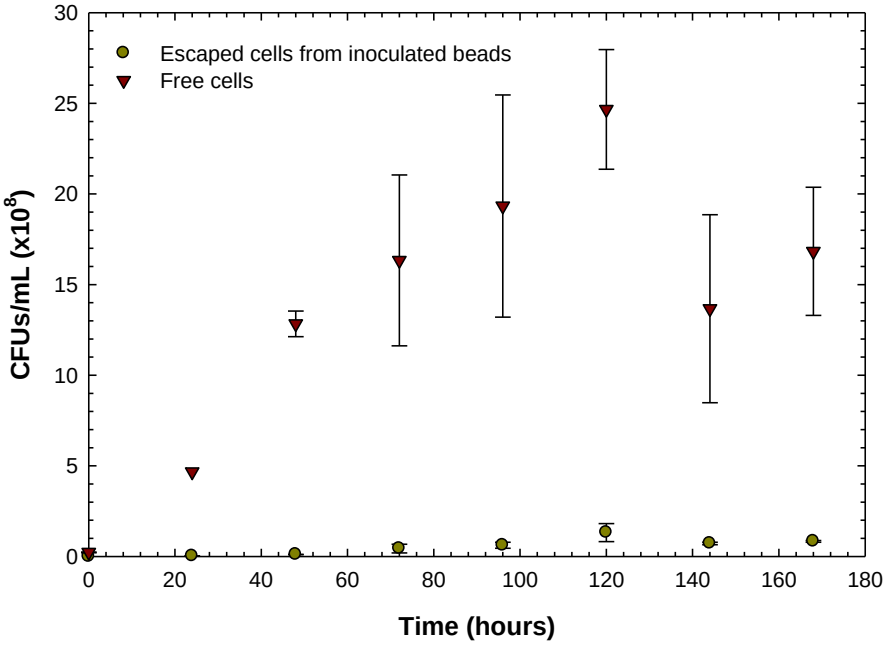


Figure 4.6. Bacterial growth in CFUs/mL for escaped and free cells.

It can be surmised that a similar dieback of cells occurred in the contaminated blank beads, leading to a similar spike in NO_3-N concentration after 144 hours. A degree of recovery in colony numbers occurred between hour 144 and 168; a 23% and 16% percent increase from colony counts after 144 hours in the free and bead treatments, respectively, coincided with a return to approximately 120 hour levels of NO_3-N (undetectable for the free cell treatment and 0.6 mg/L NO_3-N for the bead treatment). This dieback occurrence gives further evidence of the influence that the growth and metabolism of the bacteria had over remaining NO_3-N concentrations in the treatment solutions. The highest level of NO_3-N removal for the bead treatment was 95% after 96 hours and again after 120 hours; the highest level for the free cells was 100% removal after 96 hours and again after 168 hours. Average free cell treatment colony counts reached a maximum of 2.5×10^9 per mL after 120 hours. Without the occurrence of the dieback, the assimilation of NO_3-N by the bacterium is described fairly well by first order kinetics or what could be modeled as exponential decay in NO_3-N concentration (Figures 4.7 and 4.8). A nonlinear statistical regression analysis was performed using SAS® 9.3 software to compare the overall NO_3-N removal rate in the free cells to that of the beads by fitting a first order kinetic/exponential decay trend to two models via the iterative Gauss-Newton method. The full model distinguished between the treatments of free cells and beads and described the data using two separate sets of best fit parameters for an exponential decay trend. The reduced model did not consider the two separate treatments and instead used only one set of best fit parameters to describe the exponential decay trend in the entire dataset for NO_3-N removal in the test tube experiment. Table 4.1 gives the resulting equations and the nonlinear measure of quality of fit, the pseudo-R^2, for each model.

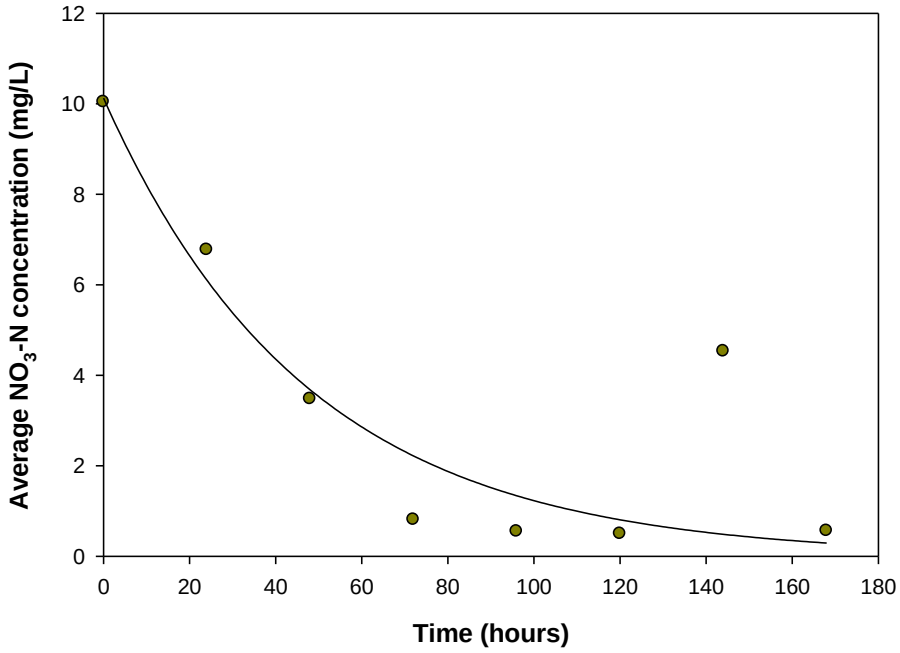


Figure 4.7. Average NO_3-N assimilation results for inoculated beads with first order kinetic/exponential decay trend line.

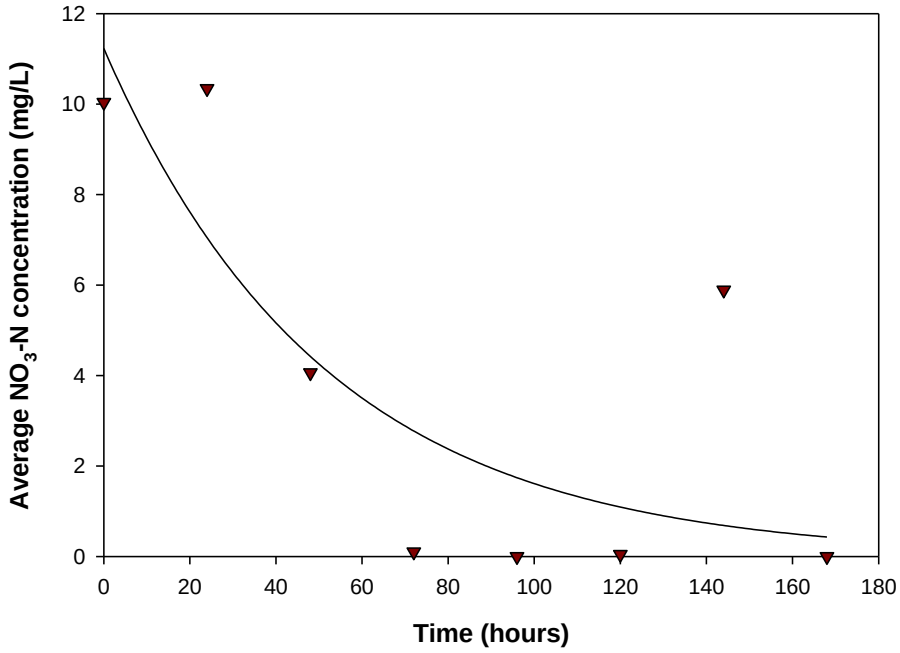


Figure 4.8. Average NO_3-N assimilation results for free cells with first order kinetic/exponential decay trend line.

Table 4.1. NO_3-N assimilation equations generated via the Gauss-Newton method, where C_t = NO_3-N concentration at time, t, C_0 = initial NO_3-N concentration, and t = time.

Model	NO_3 removal equation	Pseudo-R^2
Full		
Free cells	$C_t=C_0e^{-0.0175t}$	0.7750
Beads	$C_t=C_0e^{-0.0220t}$	0.7750
Reduced	$C_t=C_0e^{-0.0194t}$	0.7722

A sum of squares reduction test was performed to compare the quality of fit of the two models. The test found no significant difference in the quality of fit of the models, which suggests that the overall exponential decay rates in the free cells and beads did not differ to a significant degree.

4.4. Comparison of NO_3-N utilization in free and immobilized cells with aeration

In order to assure that oxygen limiting conditions in the previous test tube experiments were not altering NO_3-N utilization rates and to conduct batch experiments similar to those performed by other researchers to study denitrification rates in the laboratory, for example: Hill and Khan (2008); Song et al. (2005); and Tenokuchi et al. (2006), one liter volume reactor experiments with aeration were performed. Average temperature, average pH, average NO_3-N assimilation (mg/L NO_3-N) and normalized average NO_3-N assimilation results for the three trials performed are shown below in Figures 4.9, 4.10, 4.11 and 4.12, respectively. The free cell treatment reached a maximum average NO_3-N removal of 95.9 % after 168 hours, while the bead treatment reached its maximum average NO_3-N removal of 93.7% after 96 hours. An ANOVA analysis using SAS® 9.3 software found no significant reduction in either treatment after 72 hours.

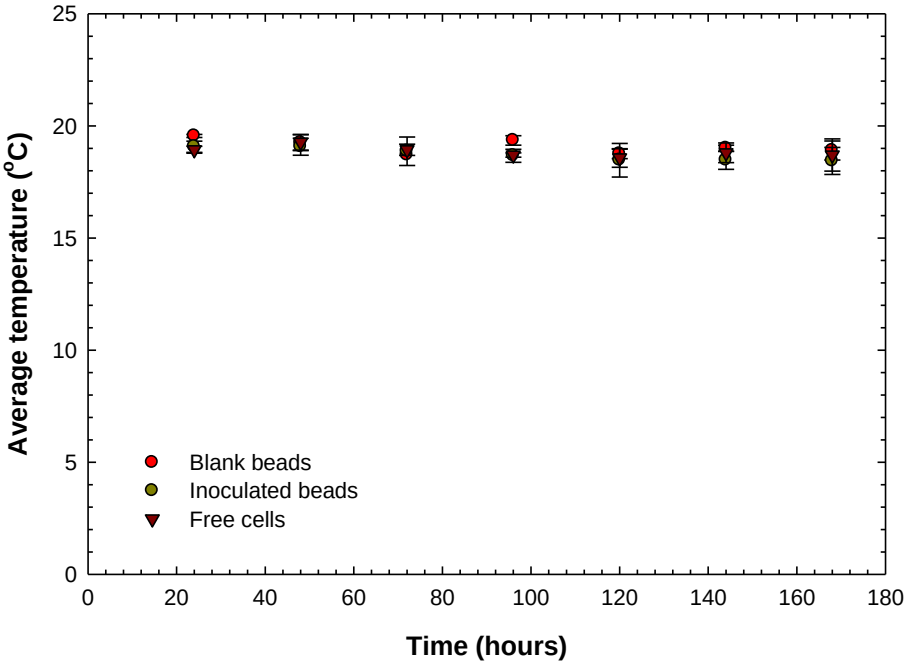


Figure 4.9. Average temperature results for batch experiment.

.

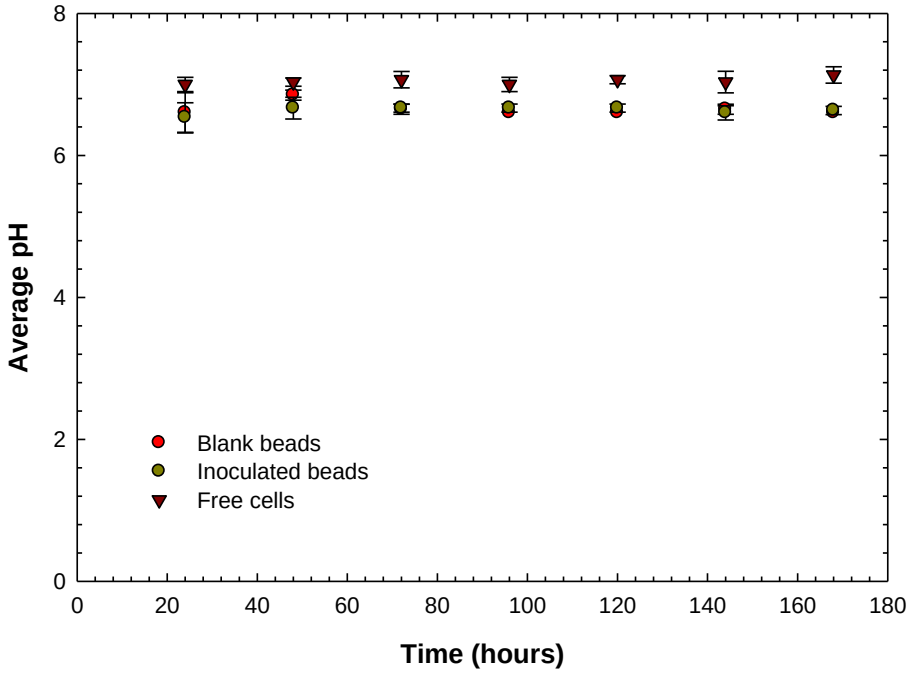


Figure 4.10. Average pH results for batch experiment.

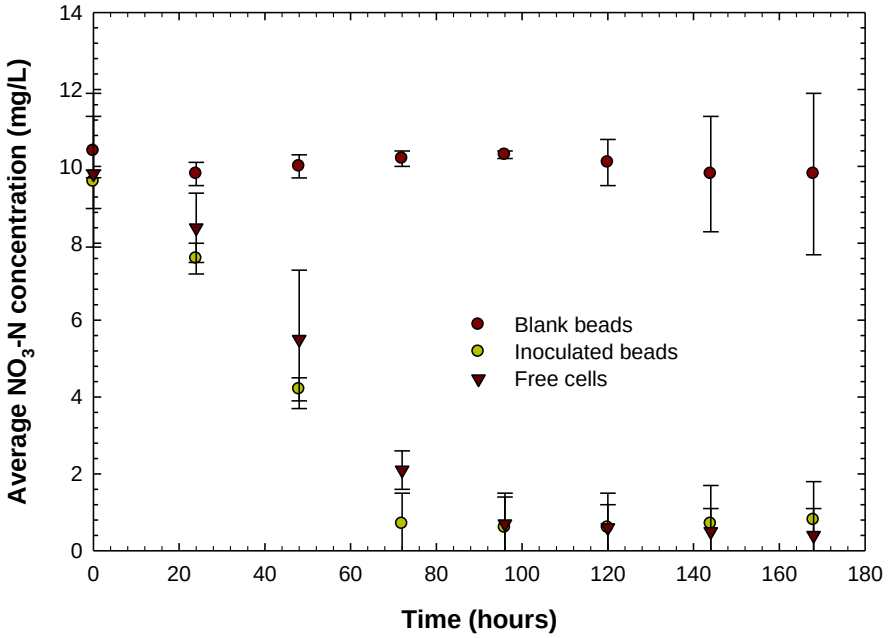


Figure 4.11. NO_3-N assimilation results for batch experiment.

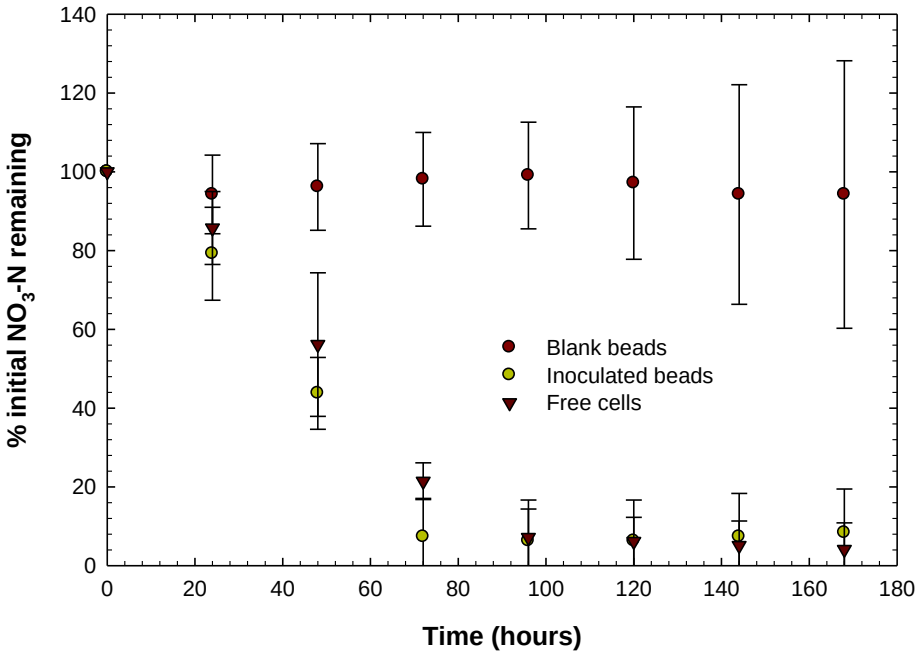


Figure 4.12. Normalized NO_3-N assimilation results for batch experiment.

A nonlinear statistical regression analysis was again performed using SAS® 9.3 software to compare the overall NO_3-N removal rate in the free cells to that of the beads by fitting a first order kinetic/exponential decay trend to both a full and reduced model via the iterative Gauss-Newton method (Figures 4.13 and 4.14). Table 4.2 gives the resulting equations and the nonlinear measure of quality of fit, the pseudo-R^2, for each model. A comparison of the full and reduced model via the sum of square reduction test found no significant difference in their quality of fit; this suggested no significant difference in the exponential decay rates of the free cells and beads under batch conditions. A comparison of the NO_3-N reduction and colony counts in trial three gives qualitative evidence of the relationship between the growth of the bacterium and the removal of NO_3-N from the reactor. As seen in Figures 4.15 and 4.16 below, the colony count of M. fujisawaense in trial three rose in contrast with the decline in NO_3-N concentration. NO_3-N reduction appears to slow with the onset of the stationary phase in the hours following the maximum colony count of 1.5 x 109 per mL after 120 hours.

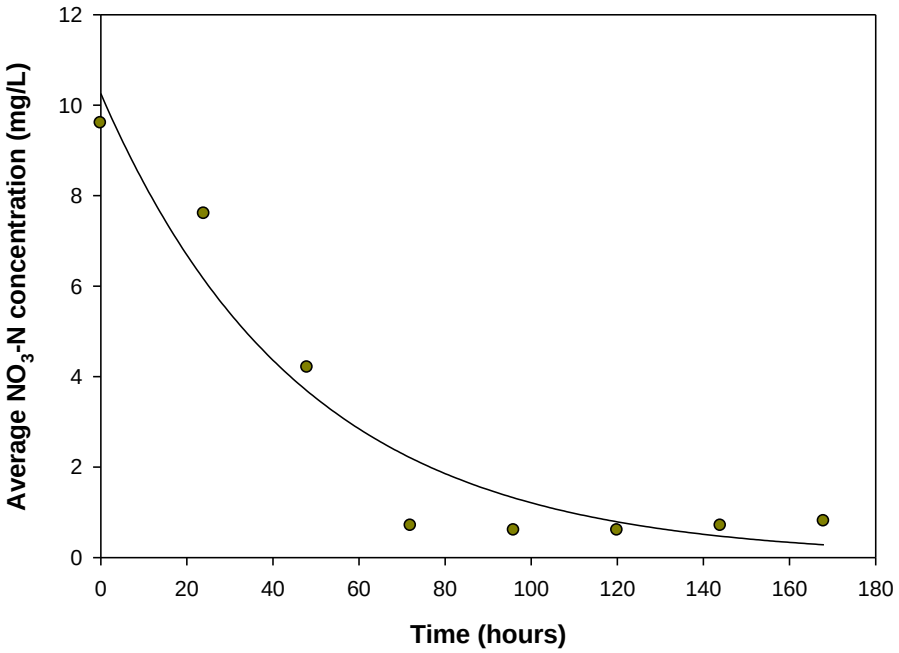


Figure 4.13. Average NO_3-N assimilation results for inoculated beads with first order kinetic/exponential decay trend line.

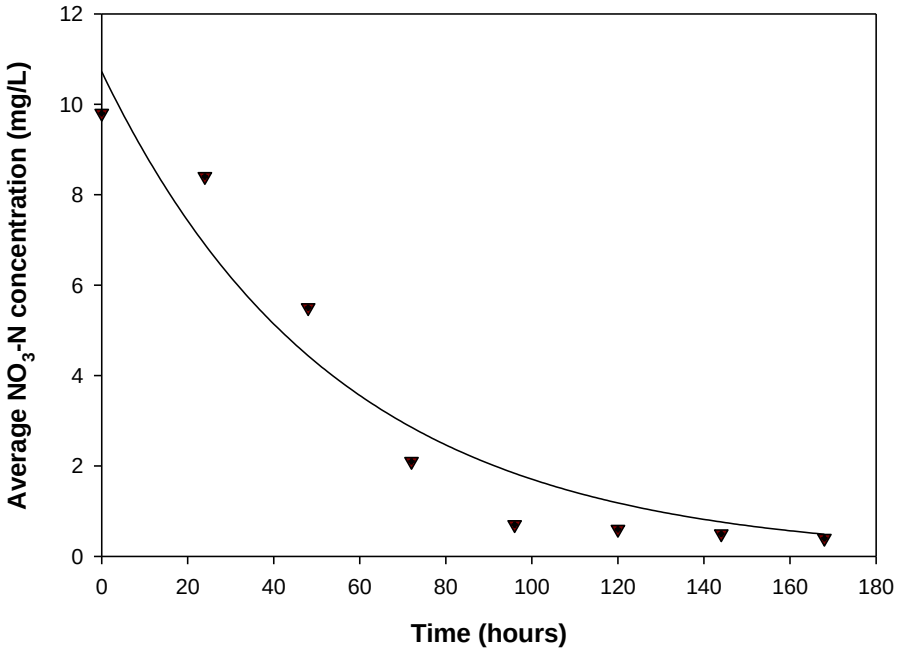


Figure 4.14. Average NO_3-N assimilation results for free cells with first order kinetic/exponential decay trend line.

Table 4.2. NO_3-N assimilation equations generated via the Gauss-Newton method, where C_t = NO_3-N concentration at time, t, C_0 = initial NO_3-N concentration, and t = time.

Model	**NO_3 removal equation**	**Pseudo-R^2**
Full		
Free cells	$C_t=C_0e^{-0.0168t}$	0.9398
Beads	$C_t=C_0e^{-0.0197t}$	0.9398
Reduced	$C_t=C_0e^{-0.0181t}$	0.9379

4.5. NO_3-N utilization in a flow-through filter experiment

A bench-scale flow through filter filled with inoculated beads was run with both the same volume of beads and concentration of NO_3-N solution as were used in the batch experiments. Average temperature, average pH, average NO_3-N assimilation, and percentage of initial NO_3-N assimilated are shown below in Figures 4.17, 4.18, 4.19, and 4.20, respectively.

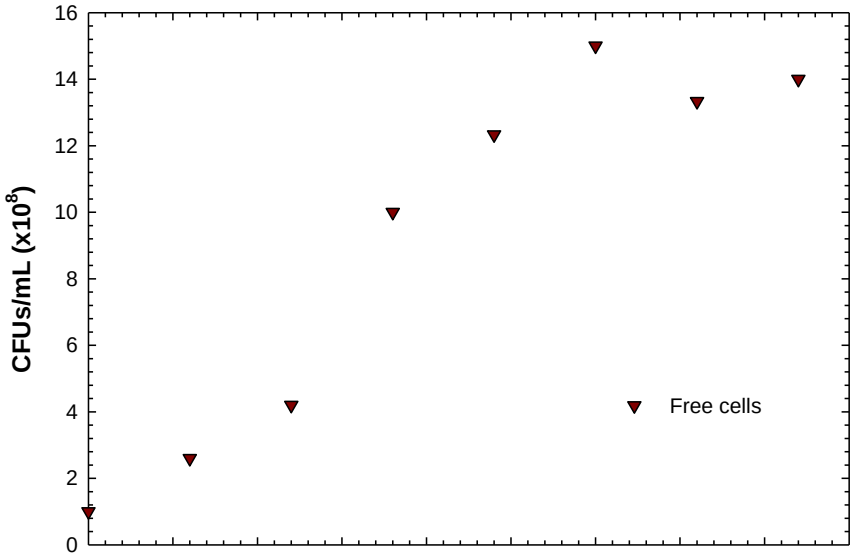


Figure 4.15. Bacterial growth in CFUs/mL for the free cell reactor of trial three of the batch experiment.

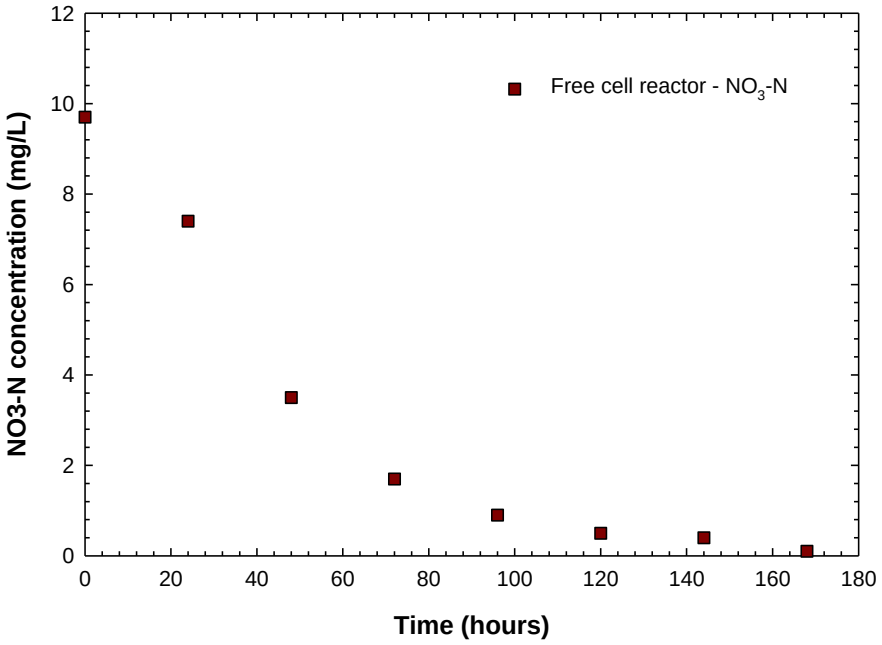


Figure 4.16. NO_3-N assimilation results for the free cell reactor of trial three of the batch experiment.

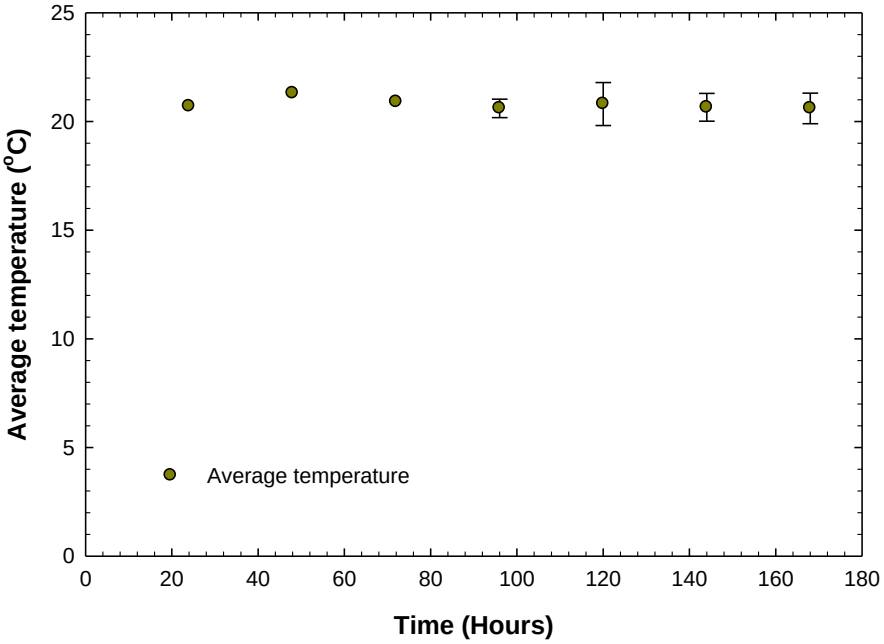


Figure 4.17. Average temperature results for flow through filter experiment.

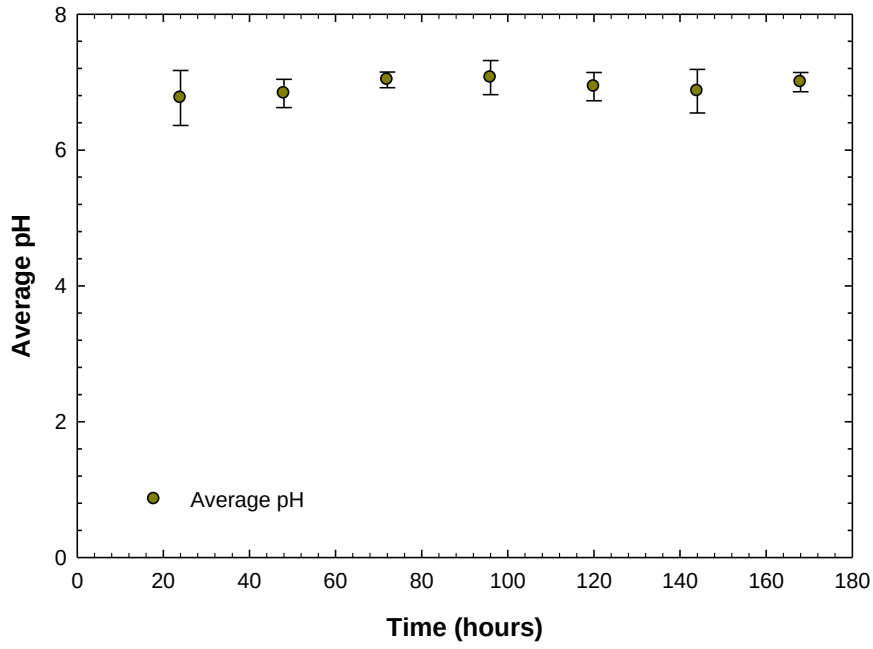


Figure 4.18. Average pH results for flow through filter experiment.

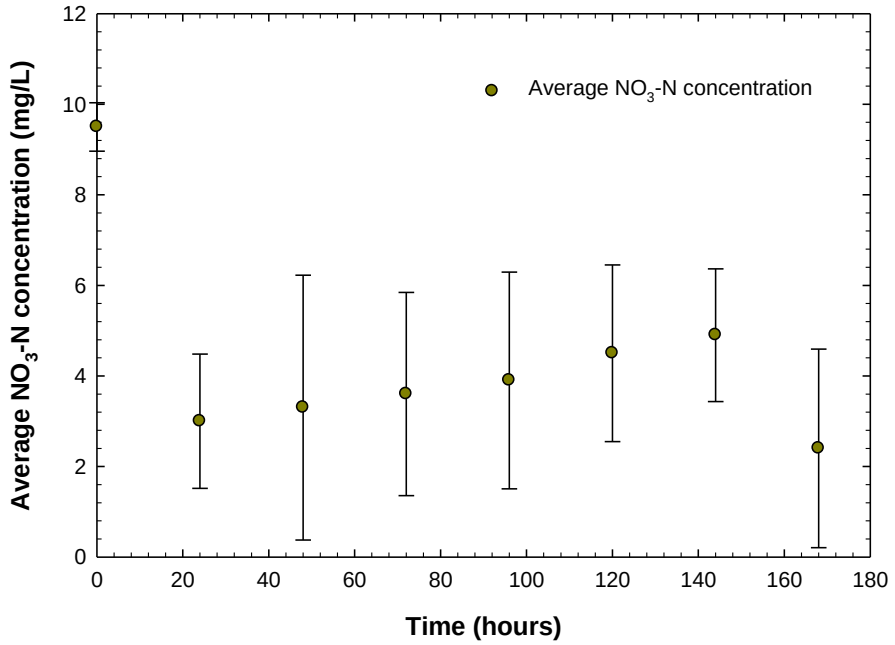


Figure 4.19. Average NO_3-N assimilation results for flow through filter experiment.

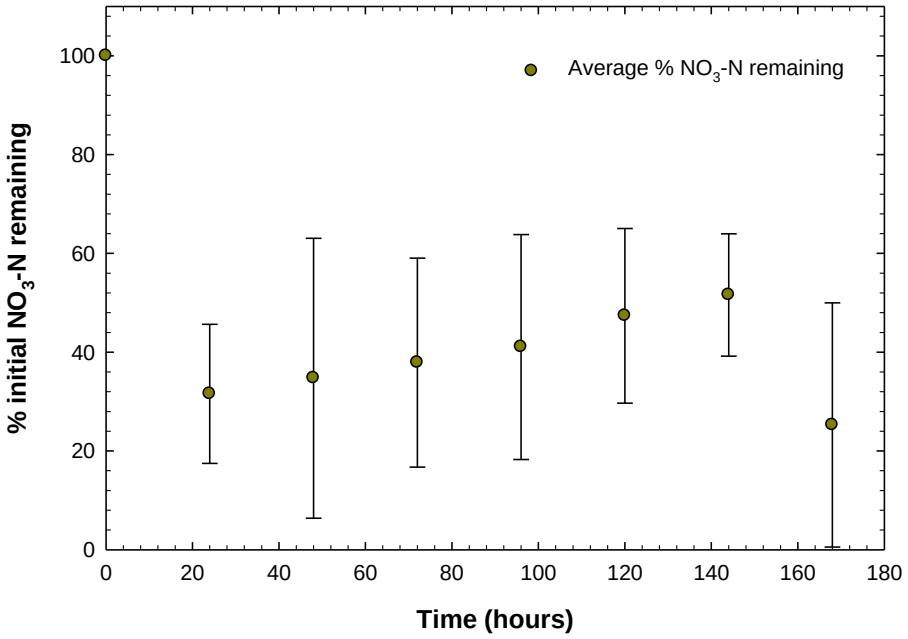


Figure 4.20. Average percentage of initial NO_3-N remaining for flow through filter experiment.

An average of 68.4% of the NO_3-N was removed from solution within the first 24 hours of the experiment. Analysis via the ANOVA and Tukey Methods at a 5% significance level using Minitab® 16 software was performed to determine if significant reduction was occurring in average nitrate concentration throughout the time period. Results showed a significant reduction in average nitrate concentration when comparing the initial average concentration to those measured after 24, 48, 72, and 168 hours. However, the average concentrations after 96, 120, and 144 hours did not significantly differ from either the initial average concentration or those measured after 24, 48, 72, and 168 hours.

CHAPTER 5. DISCUSSION

Results from the initial NO_3-N utilization testing showed free cells of *M. fujisawaense* capable of least 89.7% reduction of NO_3-N levels starting at a concentration close to the EPA's maximum contaminant level of 10 mg/L NO_3-N when using a solution containing non-limiting carbon source (methanol) and trace elements concentrations (1 and 5 g/L, respectively). A second trial to compare the NO_3-N removal of the same concentration between free cells and immobilized cells of the bacterium showed a removal of 100% of 10 mg/L NO_3-N after 96 hours in the free cell treatment, while the bead treatment showed a removal of 95% of the same NO_3-N concentration after 96 hours. The difference in the final NO_3-N concentrations between the two treatments, undetectable in the free cell treatment and 0.2 mg/L NO_3-N in the bead treatment, was minimal. Fitting a first order kinetic/exponential decay trend to both treatments with the nonlinear regression Gauss-Newton method resulted in an adequate quality of fit for both a full model that considered the treatments separately (pseudo-R^2 = 0.7750) and a reduced model that pooled all NO_3-N assimilation data together (pseudo-R^2 = 0.7722). A comparison of the two models via a sum of squares reduction test indicated no significant difference between the two, suggesting that the overall NO_3-N assimilation rates of the bead and free cell treatments did not significantly differ. The decline of NO_3-N concentrations in the blank tubes during this experiment likely occurred via a treatment tube of free cells that had been discovered to be leaking. All tubes were incubated on tube rockers in the same incubator. The presence of pink turbidity in the blanks gave visual confirmation of the contamination by *M. fujisawaense.* A bench scale reactor test was also run to test the NO_3-N removal ability of *M. fujisawaense* with aeration, more commonly used in batch reactor tests, in place of rocking on a test tube rocker. The free cell treatment reached a maximum average normalized NO_3-N removal of 95.6 % after

168 hours, while the bead treatment reached its maximum average normalized NO_3-N removal of 93.4% after 96 hours. As in the test tube experiments, the difference in lowest achieved concentrations was minimal (0.4 mg/L NO_3-N for free cells and 0.6 mg/L NO_3-N for beads). The same statistical analysis using nonlinear regression and model comparison that was used with the test tube experimental data was applied to the NO_3-N assimilation data from the batch experiments. Both the full and reduced models based on a first order kinetic/exponential decay equation resulted in a good quality of fit for the data (full model pseudo-R^2 = 0.9398 and reduced model pseudo-R^2 = 0.9379). A sum of squares reduction test resulted in a finding of no significant difference between the two models, once again suggesting that the overall rate of NO_3-N assimilation by cells freely suspended and immobilized on beads did not greatly differ. Pramanik and Khan (2009) found that the growth and substrate rate of three species of bacterial cells immobilized in calcium alginate either did not differ or were somewhat lower than the corresponding rates in freely suspended cells. Results obtained in this study for *M. fujisawaense* suggest that it may be a strain whose overall NO_3-N assimilation activity is not significantly altered by immobilization.

In a continuous flow setting of a trickle filter, immobilized *M. fujisawaense* achieved less overall reduction as compared to the batch studies conducted, reaching an average reduction that ranged from 48.4% to 74.7% of the original NO_3-N concentration. A significant reduction in average nitrate concentration occurred after 24 hours and average concentrations stayed significantly lower than the initial average until after 72 hours. Average concentrations after 96, 120, and 144 hours did not statistically differ from either the initial average or those measured after hours 24 to 72. The average concentration after 168 hours, however, was found to be statistically lower than the initial average. This may suggest that the pattern of no significant

difference in nitrate concentration after 72 hours, as seen in the batch experiments, may have been disrupted in the trickle filter between 96 and 144 hours by the combination of pump failure during a power outage that occurred sometime before hour 96 during trial two and possible bacterial desiccation issues with the trickle filter set-up. The significantly lower average after 168 hours may be due to the reset of the pump after 144 hours during trial three when it was discovered the pump had stopped delivering the solution to the filter. The reset and subsequent return of solution delivery may have caused a resurgence of nitrate uptake in the bacteria in the filter.

A comparison of NO_3-N removal by *M. fujisawaense* with that of denitrifying bacteria under laboratory conditions shows that many denitrifying bacteria are capable of higher rates of NO_3-N removal in shorter periods of time (Song et al., 2005; Tenokuchi et al., 2006; Hill and Khan, 2008; Siripattanakul et al., 2010). For example, in a study by Song et al. (2005), the denitrifier, *Ochrobactrum anthropi* SY509, reduced 100 mg/L NO_3-N to zero after two hours while immobilized on calcium alginate beads. This ability to use higher concentrations of NO_3-N in a shorter time period would be expected, however, since denitrifying bacteria are capable of utilizing NO_3-N as an electron acceptor in an energy-generating respiration process; NO_3-N assimilation, in contrast, requires energy to transform NO_3-N into nitrite, then the ammonium ion, and finally forms of organic nitrogen to be used in the cell (Bothe et al., 2007; Moreno-Vivian and Flores, 2007). But if future economic and environmental factors began to necessitate a reduction in the ever increasing introduction of additional nitrogen into the environment via the production of inorganic nitrogen fertilizers, further research of NO_3-N assimilating bacteria, or consortia of NO_3-N assimilating bacteria, like *M. fujisawaense* may be beneficial to further analyze their capability to immobilize NO_3-N and keep it in a reactive form available to living

organisms; for example, technologies such as trickle filters or bio-reactors may be applied to recovering leached NO_3-N from agricultural water and returning the nitrogen to the fields in the form of composted biosolids for organic farming. This would require further research of the ability of NO_3-N immobilizers to grow and take up NO_3-N under different environmental conditions of temperature, pH, and initial concentrations of NO_3-N; rates of remineralizaton of biomass back into inorganic forms of nitrogen and what range of carbon sources are utilized by the organisms would also need to be accessed. Another potential focus for contaminant biodegrading organisms like those in the genus, *Methylobacterium,* may be studying their bioremediation potential to see if, for example, there are members that are naturally or, via horizontal gene transfer, could become capable of simultaneously biodegrading co-contaminants of NO_3-N while utilizing the NO_3-N as a nitrogen source. Additionally, species with a wide range of tolerance for different contaminants and environmental conditions, like the strain of *Methylobacterium fujisawaense* described in De Marco, et al. (2004), could be assessed for use in dual augmentation, a bioremediation process that involves the introduction of a sensitive contaminant degrading organism with a widely tolerant partner organism; such pairings have been shown to improve the overall biodegradation process (De Marco, et al., 2004). A dual augmentation process with simultaneous NO_3-N removal could prove useful in some contaminated areas.

An interesting observation occurred when plans to monitor the growth rate of the free cells in the batch experiment by spectrophotometer readings of OD_{600} were disrupted upon an unexpected rise in OD_{600} values above the range determined during growth curve construction for the organism. To determine if the bacteria had grown faster than in the original growth curve construction, CFUs/mL were measured daily by the dilution series plating during the third trial

run. Resulting CFUs/mL were not as many as would have been expected with the higher OD_{600} readings when compared to the constructed growth curve. A possible explanation may be that a similar phenomenon of increased pigment production under nutrient limited conditions was occurring in the treatments of *M. fujisawaense* like in cultures of *Methylobacterium extorquens* (previously *Pseudomonas extorquens*) by Downs and Harrison (1974). In their study, limited magnesium levels in the presence of non-carbon limiting conditions led to an increase of carbon used for oxo-carotenoid production. They also hypothesized that a limitation in any nutrient with no corresponding limitation in carbon source could possibly give rise to increased pigment production. This type of response to limited NO_3-N levels in the minimal solution used here may have caused an increase in pigmentation production in *M. fujisawaense*, thus explaining the higher OD_{600} values paired with less than expected colony counts.

CHAPTER 6. CONCLUSION

The NO_3-N assimilating bacterium, *M. fujisawaense,* was found to be capable of 89 to 100% removal of the EPA Maximum Contaminant Level (10 mg/L NO_3-N) of NO_3-N as freely suspended cells under aerobic, batch experiment conditions. When immobilized on calcium alginate beads under these conditions, the bacteria achieved NO_3-N removal rates of approximately 93 to 95% in either the same amount of time or one day earlier than their free cell counterparts. The results and statistical analysis suggest that the immobilization of *M. fujisawaense* on calcium alginate beads does not have a deleterious impact on its ability to immobilize NO_3-N under batch experiment conditions. The potential benefits of use of such NO_3-N assimilating bacteria for the bioremediation of NO_3-N in water include a recovery of nitrogen in a form accessible to living organisms, such as crop plants. A second potential benefit, with respect to specific contaminant resistant or biodegrading organisms, would be the ability to bioremediate NO_3-N and co-contaminants simultaneously. Further study of *M. fujisawaense* may be warranted to explore the possibility of its use to remove both NO_3-N and other water contaminants simultaneously via assimilation and biodegradation.

REFERENCES

Anandham, R.; Indiragandhi, P.; Madhaiyan, M.: Chung, J.; Yul Ryu, K.; Jin Jee, H.; Sa, T. (2009) Thiosulfate Oxidation and Mixotrophic Growth *of Methylobacterium goesingense* and *Methylobacterium fujisawaense*. *J. Microbiol. Biotechnol.*, 19, 17- 22.

American Type Culture Collection (2011) Product Information Sheet for ATCC® 35065™.

Avery, A. A. (1999) Infantile Methemoglobinemia: Reexamining the Role of Drinking Water Nitrates. *Environ. Health Perspect.*, 107, 1 – 8.

Bengtson, P.; Bengtsson, G. (2005) Bacterial Immobilization and Remineralization of N at Different Growth Rates and N Concentrations. *FEMS Microbiol. Ecol.*, 54, 13 – 19.

Bothe, H.; Ferguson, S. J.; Newton, W. E., eds. (2007) *Biology of the Nitrogen Cycle*. Elsevier: Oxford, UK.

Breisha, G. Z.; Winter, J. (2010) Bio-Removal of Nitrogen from Wastewaters-A Review. *J. Am. Sci*., 6, 508 - 528.

Chistosserdova, L.; Laukel, M.; Portais, J. C.; Vorholt, J. A.; Lidstrom, M.E. (2004) Multiple Formate Dehydrogenase Enzymes in the Facultative Methylotroph *Methylobacterium extorquens* AM1 are Dispensable for Growth on Methanol. *J. Bacteriol*., 186, 22 - 28.

Corpe, W. A.; Rheem, S. (1989) Ecology of the Methylotrophic Bacteria on Living Leaf Surfaces. *FEMS Microbiol. Lett*., 62, 243 - 249 (abstract).

Dalton, H.; Brand-Hardy, R. (2003) Nitrogen: the Essential Public Enemy. *J. Appl. Ecol*., 40, 771 - 781.

De Marco, P; Pacheco, C. C.; Figueiredo, A. R.; Moradas-Ferreira, P. (2004) Novel Pollutant-Resistant Methylotrophic Bacteria for Use in Bioremediation. *FEMS Microbiol. Lett.*, 234, 75 - 80.

Downs, J.; Harrison, D. E. (1974) Studies on the Production of Pink Pigment in *Pseudomonas extorquens* NCIB 9399 Growing in Continuous Culture. *J. Appl. Bacteriol*., 37, 65 - 74.

Dubrovsky, N.M.; Burow, K. R.; Clark, G. M.; Gronberg, J. M.; Hamilton, P. A.; Hitt, K. J.: Mueller, D. K.; Munn, M. D.; Nolan, B. T.; Puckett, L. J.; Rupert, M. G.; Short, T. M.; Spahr, N. E.; Sprague, L. A.; Wilber, W. G. (2010) *The Quality of our Nation's Waters—Nutrients in the Nation's Streams and Groundwater, 1992–2004*. Circular 1350; US Geological Survey: Reston, VA.

US Environmental Protection Agency (2007) *Wastewater Management Fact Sheet: Denitrifying Filters;* EPA-832-F-07-014; Washington, D.C.

US Environmental Protection Agency (2012) *Basic Information about Nitrate in Drinking Water.* Available at http://water.epa.gov/drink/contaminants/basicinformation/nitrate.cfm. Accessed Sep 17, 2012.

Ergas, S. J.; Reuss, A. F. (2001) Hydrogenotrophic Denitrification of Drinking Water Using a Hollow Fibre Membrane Bioreactor. *J. Water Supply Res. T.*, 50, 161-171.

Fan, A. M.; Steinberg, V. E. (1996) Health Implications of Nitrate and Nitrite in Drinking Water: An Update on Methemoglobinemia Occurrence and Reproductive and Developmental Toxicity. *Regul. Toxicol. Pharmacol.*, 23, 35 - 43.

Fewtrell, L. (2004) Drinking-Water Nitrate, Methemoglobinemia, and Global Burden of Disease: A Discussion. *Environ. Health Perspect.*, 112, 1371-1374.

Fields, S. (2004) Global Nitrogen: Cycling Out of Control. *Environ. Health Perspect.*, 112, A557 – A563.

Galloway, J.T.; Aber, J. D.; Erisman, W.; Seitzinger, S. P.; Howarth, W.; Cowling, E. B.; Cosby, B. J. (2003) The Nitrogen Cascade. *BioScience,* 53, 341 - 356.

Garrity, G.M.; Bell, J. A.; Lilburn, T. (2005) Family IX: Methylobacteriaceae fam. nov. Pages

567-571 *in* Brenner, D. J.; Krieg, N. R.; Staley, J. T., eds. *Bergey's Manual of Systematic Bacteriology*. Vol. 2 Pt. C. Springer: New York.

Gombotz, W.R.; Wee, S. F. (1998) Protein Release from Alginate Matrices. *Adv. Drug Delivery Rev.*, 31, 267 - 285.

Hill, C.B.; Khan, E. (2008) A Comparative Study of Immobilized Nitrifying and Co-Immobilized Nitrifying and Denitrifying Bacteria for Ammonia Removal from Sludge Digester Supernatant. *Water, Air, Soil Pollut.*, 195, 23 – 33.

Hiraishi, A.; Furuhata, K.; Matsumoto, A.; Koike, K. A.; Fukuyama, M.; Tabuchi, K. (1995) Phenotypic and Genetic Diversity of Chlorine-Resistant *Methylobacterium* Strains Isolated from Various Environments. *Appl. Environ. Microbiol*. 61, 2099 - 2107.

Idris, R.; Kuffner, M.; Bodrossy, L.; Puschenreiter, M.; Monchy, S.; Wenzel, W. W.; Sessitsch, A. (2006) Characterization of Ni-Tolerant Methylobacteria Associated with the Hyperaccumulating Plant *Thlaspi goesingense* and Description of *Methylobacterium goesingense* sp. nov. *Syst. Appl. Microbiol*. 29, 634 - 644.

Kelso, B.H.L.; Smith, R.V.; Laughlin, R.J. (1999) Effects of Carbon Substrates on Nitrite Accumulation in Freshwater Sediments. *Appl. Environ. Microbiol.*, 65, 61 – 66.

Kim, Y.K.; Park, Y.J.; Song, S.H.; Yoo, Y.J. (2007) Nitrate Removal without Carbon Source Feeding by Permeabilized *Ochrobactrum anthropi* SY509 Using an Electrochemical Bioreactor. *Enzyme Microb. Technol*. 41, 663 – 668.

Knobeloch, L.; Salna, B.; Hogan, A.; Postle, J.; Anderson, H. (2000) Blue Babies and Nitrate-Contaminated Well Water. *Environ. Health Perspect*., 108, 675 – 678.

Kobayashi, M.; Ishimoto, M. (1973) Aerobic Inhibition of Nitrate Assimilation in *Escherichia coli. J. Basic Microbiol.*, 13, 405 – 413.

Kongshaug, G. (1998) Energy Consumption and Greenhouse Gas Emissions in Fertilizer Production. *Proceedings of the IFA Technical Conference*; Marrakesh, Morocco, Sept 28 – Oct 1; 1 – 18.

Konsoula, Z.; Liakopoulou-Kyriakides, M. (2006) Thermostable α-amylase Production by *Bacillus subtilis* Entrapped in Calcium Alginate Gel Capsules. *Enzyme Microb. Technol*., 39, 690 - 696.

Korom, S.F. (1992) Natural Denitrification in the Saturated Zone: a Review. *Water Resour. Res.*, 28, 1657 - 1668.

Kutschera, U.; Koopmann, V. (2005) Growth in Liverworts of the Marchantiales is Promoted by Epiphytic Methylobacteria. *Naturwissenschaften* 92, 347 - 349 (abstract).

Lambert, K.F.; Driscoll, C. (2003) *Nitrogen Pollution: From the Sources to the Sea*. Hubbard Brook Research Foundation: Hanover, NH.

L'hirondel, J.L.; Avery, A. A.; Addiscott, T. (2006) Dietary Nitrate: Where is the risk? *Environ. Health Perspect.*, 114, A458 - A459.

Lin, J.T.; Stewart, V. (1998) Nitrate Assimilation by Bacteria. Pages 1-30 *in* Poole, R. K. ed. *Advances in Microbial Physiology*. Vol. 39. Academic Press: San Diego, CA.

Liu, S. X. (2007) *Food and Agricultural Wastewater Utilization and Treatment*. Blackwell Publishing: Ames, IA.

Madhaiyan, M.; Poonguzhali, S. (2006) Regulation of Ethylene Levels in Canola (*Brassica campestris*) by 1-Aminocyclopropane-1-Carboxylate Deaminase-Containing *Methylobacterium fujisawaense*. *Planta*, 224, 268 - 278.

Madhaiyan, M.; Kim, B. Y.; Poonguzhali, S.; Kwon, S. W.; Song, M. H.; Ryu, J. H.; Go, S. J.; Koo, B. S.; Sa, T. M. (2007) *Methylobacterium oryzae* sp. nov., an Aerobic, Pink-

Pigmented, Facultatively Methylotrophic, 1-Aminocyclopropane-1-Carboxylate Deaminase-Producing Bacterium Isolated from Rice. *Int. J. Syst. Evol. Microbiol.,* 57, 326 - 331.

Madigan, M.T.; Martinko, J.M. (2005) *Brock Biology of Microorganisms*, 11th ed. Benjamin Cummings: Boston. 1088 p.

Madigan, M.T.; Martinko, J.M.; Stahl, D.A.; Clark, D.P. (2012) *Brock Biology of Microorganisms,* 13th ed. Benjamin Cummings: Boston. 1152 p.

Metcalf and Eddy, Inc. (1991) Tchobanoglous, G.; Burton, F. L., eds. *Wastewater Engineering: Treatment, Disposal, and Reuse*. 3rd ed. McGraw-Hill, Inc.: New York. 1334 p.

Mo, K.; Lora, C. O.; Wanken, A. E.; Javanmaridan, M.; Yang, X.; Kulpa, C. F. (1997) Biodegradation of Methyl t-Butyl Ether by Pure Bacterial Cultures. *Appl. Microbiol. Biotechnol.*, 47, 69 - 72.

Moreno-Vivian, C.; Flores, E. (2007) Nitrate Assimilation in Bacteria. Pages 263-282 *in* Bothe, H.; Ferguson, S. J.; Newton, W. E., eds. *Biology of the Nitrogen Cycle*. Elsevier: Oxford, UK.

Munch, J.C.; Velthof, G. L. (2007) Denitrification and Agriculture. Pages 331-342 *in* Bothe, H.; Ferguson, S. J.; Newton, W. E., eds. *Biology of the Nitrogen Cycle*. Elsevier: Oxford, UK.

Patt, T.E.; Cole, G. C.; Hanson, R. S. (1976) *Methylobacterium,* a New Genus of Facultatively Methylotrophic Bacteria. *Int. J. Syst. Bacteriol*., 26, 226 - 229.

Pramanik, S.; Khan, E. (2008) Effects of Cell Entrapment on Growth Rate and Metabolic Activity of Mixed Cultures in Biological Wastewater Treatment. *Enzyme Microb. Technol*., 43, 245 - 251.

Pramanik, S.; Khan, E. (2009) Effects of Cell Entrapment on Growth Rate and Metabolic Activity of Pure Cultures Commonly found in Biological Wastewater Treatment. *Biochem. Eng. J.,* 46, 286 - 293.

Rezaee, A.; Godini, H.; Bakhtou, H. (2008) Microbial Cellulose as Support Material for the Immobilization of Denitrifying Bacteria. *Environ. Eng. Manage. J.,* 7, 589 - 594.

Ravishankara, A. R.; Daniel, J. S.; Portmann, R. W. (2009) Nitrous Oxide (N_2O): The Dominant Ozone-Depleting Substance Emitted in the 21st Century. *Science,* 326, 123 – 125.

Rivett, M.O.; Buss, S.R.; Morgan, P.; Smith, J.W.N.; Bemment, C.D. (2008) Nitrate Attenuation in Groundwater: A Review of Biogeochemical Controlling Processes. *Water Res. (IWA),* 42, 4215 – 4232.

Robertson, G.P.; Paul, E. A.; Harwood, R. R. (2000) Greenhouse Gases in Intensive Agriculture: Contributions of Individual Gases to the Radiative Forcing of the Atmosphere. *Science,* 289, 1922 - 1925.

Rosiak, J.M.; Janik, I.; Kadlubowski, M.; Kozicki, P.; Kujawa, P.; Stasica, P.; Ulanski, P. (2002) Radiation Formation of Hydrogels for Biomedical Applications. Available at http://www.mitr.p.lodz.pl/biomat/raport/book_index.html. Accessed: May 22, 2013.

Rossetto, P.B.; Dourado, M. N.; Quecine, M. C.; Andreote, F. D.; Araujo, W. L.; Azevedo, J. L.; Pizzirani-Kleiner, A. A. (2011) Specific Plant Induced Biofilm Formation in *Methylobacterium* Species. *Braz. J. Microbiol*., 42, 878 - 883.

Rowland, F. S. (2006) Stratospheric Ozone Depletion. *Phil. Trans. R. Soc. B.,* 361, 769 - 790.

Schauer, S.; Kampfer, P.; Wellner, S.; Sproer, C.; Kutschera, U. (2011) *Methylobacterium marchantiae* sp. nov., a Pink Pigmented, Facultatively Methylotrophic Bacterium Isolated from the Thallus of a Liverwort. *Int. J. Syst. Evol. Microbiol.,* 61, 870 - 876.

Schipper, L.A.; Vojvodic-Vukovic, M. (2000) Nitrate Removal from Groundwater and Denitrification Rates in a Porous Treatment Wall Amended with Sawdust. *Ecol. Eng.*, 14, 269 – 278.

Schrader, J.; Schilling, M.; Holtmann, D.; Sell, D.; Filho, M. V.; Marx, A.; Vorholt, J. A. (2008) Methanol-Based Industrial Biotechnology: Current Status and Future Perspectives of Methylotrophic Bacteria. *Trends Biotechnol.*, 27, 107 - 115.

Shrimali, M.; Singh, K. P. (2001) New Methods of Nitrate Removal from Water. *Environ. Pollut.*, 112, 351 - 359.

Siripattanakul, S.; Pochant, C. J.; Khan, E. (2010) Nitrate Removal from Agricultural Infiltrate by Bioaugmented Free and Alginate Entrapped Cells. *Water Environ. Res*. 82, 617 - 621.

Song, S.H.; Choi, S. S.; Park, K.; Young, J. Y. (2005) Novel Hybrid Immobilization of Microorganisms and its Applications to Biological Denitrification. *Enzyme Microb. Technol.*, 37, 567 – 573.

Strovas, T.J; Lidstrom, M. E. (2009) Population Heterogeneity in *Methylobacterium extorquens* AM1. *Microbiology* (Reading, U. K.), 155, 2040 - 2048.

Takaya, N.; Catalan-Sakairi, M. A. B.; Sakaguchi, Y.; Kato, I.; Zhou, Z.; Shoun, H. (2003) Aerobic Denitrifying Bacteria that Produce Low Levels of Nitrous Oxide. *Appl. Environ. Microbiol*., 69, 3152 - 3157.

Tenokuchi, D.; Yoshida, M.; Hatanaka, C.; Toorisaka, E.; Hatate, Y. (2006) Core-Shell Microbioreactor Microencapsulated Denitrifying Bacteria for Nitrate-Nitrogen Treatment. *Polym. Bull*., 56, 275 – 284.

Trotsenko, Y.A.; Ivanova, E. G.; Doronina, N. V. (2001) Aerobic Methylotrophic Bacteria as

Phytosymbionts. *Microbiology* (Reading, U. K.), 70, 725 - 736.

Ulehlova, B. (1988) The Nitrogen Cycle. Pages 406 – 469 *in* Vancura, V.; Kunc, F., eds. *Soil Microbial Associations: Control of Structures and Functions.* Elsevier: New York, NY.

Urakami, T.; Araki, H.; Suzuki, K. I.; Komagata, K. (1993) Further Studies of the Genus *Methylobacterium* and Description of *Methylobacterium aminovorans* sp. *nov. Int. J. Syst. Bacteriol*., 43, 504 - 513.

Van Aken, B.; Peres, C. M.; Lafferty Doty, S.; Yoon, J. M.; Schnoor, J. L. (2004a) *Methylobacterium populi* sp. nov., a Novel Aerobic, Pink-Pigmented, Facultatively Methylotrophic, Methane-Utilizing Bacterium Isolated from Poplar Trees (*Populus deltoids* x *nigra* DN34). *Int. J. Syst. Evol. Microbiol*., 54, 1191 - 1196.

Van Aken, B.; Yoon, J. M.; Schnoor, J. L. (2004b) Biodegradation of Nitro-Substituted Explosives 2,4,6-Trinitrotoluene, Hexahydro-1,3,5-Trinitro-1,3,5-Triazine, and Octahydro-1,3,5,7-Tetranitro-1,3,5-Tetrazocine by a Phytosymbiotic *Methylobacterium* sp. Associated with Poplar Tissues (*Populus deltoides* x *nigra* DN34). *Appl. Environ. Microbiol.,* 70, 508 - 517.

Van Dien, S.J.; Okubo, Y.; Hough, M. T.; Korotkova, N.; Taitano, T.; Lidstrom, M. E. (2003) Reconstruction of C3 and C4 Metabolism in *Methylobacterium extorquens* AM1 Using Transposon Mutagenesis. *Microbiology* (Reading, U. K.), 149, 601 - 609.

Van Dommelen, A.; Vanderleyden, J. (2007) Associative Nitrogen Fixation. Pages 179-192 *in* Bothe, H.; Ferguson, S. J.; Newton, W. E., eds. *Biology of the Nitrogen Cycle*. Elsevier: Oxford, UK.

Vasiliadou, I.A.; Tziotzios, G.; Vayenas, D. V. (2008) A Kinetic Study of Combined Aerobic Biological Phenol and Nitrate Removal in Batch Suspended Growth Cultures. *Int.*

Biodeterior. Biodegrad., 61, 261 - 271.

Vitousek, P.M.; Aber, J. D.; Howarth, R. W.; Liken, G. E.; Matson, P. A.; Schindler, D. W.; Schlesinger, W. H.; Tilman, D. G. (1997) Human Alteration of the Global Nitrogen Cycle: Sources and Consequences. *Ecol. Applic.* 7, 737 - 750.

Walton, G. (1951) Survey of Literature Relating to Infant Methemoglobinemia due to Nitrate-Contaminated Water. *Am. J. Public Health*, 41, 986 – 996.

Wang, Q.; Feng, C.; Zhao, Y.; Hao, C. (2009) Denitrification of NO_3-N Contaminated Groundwater with a Fiber-Based Biofilm Reactor. *Bioresour. Technol.,* 100, 2223 - 2227.

Ward, M.H.; Cerhan, J. R.; Colt, J. S.; Hartge, P. (2006) Risk of Non-Hodgkin Lymphoma and NO_3-N and Nitrite from Drinking Water and Diet. *Epidemiology*, 17, 375 - 382.

Ward, M.H.; DeKok, T. M.; Levallois, P.; Brender, J.; Gulis, G.; Nolan, B. T.; VanDerslice, J. (2005) Workgroup Report: Drinking-Water NO_3-N and Health—Recent Findings and Research Needs. *Environ. Health Perspect.,* 113, 1607 – 1614.

Ward, M.H.; Mark, S. D.; Cantor, K. P.; Weisenburger, D. D; Correa-Villasenor. A.; Zahm, S. H. (1996) Drinking Water Nitrate and the Risk of Non-Hodgkin's Lymphoma. *Epidemiology* 7, 465 - 471.

World Health Organization. (2011) *Guidelines for Drinking Water Quality, 4th Ed*. World Health Organization: Geneva, Switzerland.

Wood, S.; Cowie, A. (2004) A Review of Greenhouse Gas Emission Factors for Fertiliser Production. Available at http://www.leaftc.co.uk/downloads/cc/GHG_Emission_Fertilizer_Production_June2004.pdf. Accessed: February 20, 2013.

Wrage, N.; Velthof, G. L.; Van Beusichem, M. L.; Oenema, O. (2001) Role of Nitrifier Denitrification in the Production of Nitrous Oxide. *Soil Biol. Biochem.,* 33, 1723-1732.

Zhang, P.; Lapara, T. M.; Goslan, E. H.; Xie, Y.; Parsons, S.A.; Hozalski, R. M. (2009) Biodegradation of Haloacetic Acids by Bacterial Isolates and Enrichment Cultures from Drinking Water Systems. *Environ. Sci. Technol.,* 43, 3169 - 3175.

APPENDIX A. NO_3-N UTILIZATION EXPERIMENT DATA TABLES AND CHROMATOGRAMS

Table A.1. Sample code table for NO_3-N utilization samples.

Control Samples	**Code**
NO_3-N only control, replicate 1	stk 1 (a -c)
NO_3-N only control, replicate 2	stk 2 (a-c)
NO_3-N/methanol/trace element solution control, replicate 1	blk 1 (a-c)
NO_3-N/methanol/trace element solution control, replicate 2	blk 2 (a-c)
Treatment Samples	
NO_3-N/methanol/bacteria treatment, replicate 1	s 1 (a-c)
NO_3-N/methanol/bacteria treatment, replicate 2	s 2 (a-c)
NO_3-N/methanol/trace element/bacteria treatment, replicate 1	s w TE 1 (a-c)
NO_3-N/methanol/trace element/bacteria treatment, replicate 2	s w TE 2 (a-c)
Reservoir Samples	
NO_3-N only stock solution, refrigerated	R
Standard	
Dionex® Seven Anion Standard	7A

Table A.2. NO_3-N utilization experiment NO_3-N concentrations and corresponding chromatogram locations.

Sample Code	Hour	NO_3-N(mg/L)	Chromatogram Appendix #	Appendix page #
7A	48	22.6	Figure A.1 (starting with 7A in the top left, moving left to right down the page, ending with stk 2b in the bottom right)	64
stk 1a	48	8.4		
stk 1b	48	8.5		
stk 1c	48	7.8		
stk 2a	48	8.3		
stk 2b	48	8.2		
stk 2c	48	8.1	Figure A.2 (starting with stk 2c in the top left, moving left to right down the page, ending with blk 2b in the bottom right)	65
blk 1a	48	8.1		
blk 1b	48	7.5		
blk 1c	48	8.4		
blk 2a	48	8.1		
blk 2b	48	8.2		
blk 2c	48	7.8	Figure A.3 (starting with blk 2c in the top left, moving left to right down the page, ending with s 2b in the bottom right)	66
s 1a	48	10.2		
s 1b	48	8.2		
s 1c	48	8.8		
s 2a	48	8.0		
s 2b	48	8.2		
s 2c	48	8.5	Figure A.4 (starting with s 2c in the top left, moving left to right down the page, ending with s w TE 2b in the bottom right)	67
s w TE 1a	48	5.6		
s w TE 1b	48	7.2		
s w TE 1c	48	6.6		
s w TE 2a	48	7.7		
s w TE 2b	48	7.0		
s w TE 2c	48	8.6	Figure A.5 (starting with s w TE 2c in the top left, moving left to right down the page, ending with stk 2a in the bottom right)	68
7A	72	22.6		
stk 1a	72	8.1		
stk 1b	72	8.5		
stk 1c	72	7.7		
stk 2a	72	8.2		
stk 2b	72	7.8	Figure A.6 (starting with stk 2b in the top left, moving left to right down the page,	69

(continued)

Table A.2. NO_3-N utilization experiment NO_3-N concentrations and corresponding chromatogram locations (continued).

Sample Code	Hour	NO_3-N(mg/L)	Chromatogram Appendix #	Appendix page #
stk 2c	72	8.0	ending with blk 2a in the bottom right)	69
blk 1a	72	8.0		
blk 1b	72	8.1		
blk 1c	72	8.2		
blk 2a	72	8.1		
blk 2b	72	8.0	Figure A.7 (starting with blk 2b in the top left, moving left to right down the page, ending with s 2a in the bottom right)	70
blk 2c	72	8.0		
s 1a	72	9.5		
s 1b	72	9.1		
s 1c	72	9.0		
s 2a	72	9.5		
s 2b	72	9.7	Figure A.8 (starting with s 2b in the top left, moving left to right down the page, ending with s w TE 2a in the bottom right)	71
s 2c	72	9.9		
s w TE 1a	72	6.3		
s w TE 1b	72	5.6		
s w TE 1c	72	6.1		
s w TE 2a	72	6.1		
s w TE 2b	72	4.8	Figure A.9 (starting with s w TE 2b in the top left, moving left to right down the page, ending with stk 1c in the bottom right)	72
s w TE 2c	72	7.2		
7A	96	22.6		
stk 1a	96	8.3		
stk 1b	96	8.3		
stk 1c	96	8.0		
stk 2a	96	8.8	Figure A.10 (starting with stk 2a in the top left, moving left to right down the page, ending with blk 1c in the bottom right)	73
stk 2b	96	8.4		
stk 2c	96	8.6		
blk 1a	96	7.6		
blk 1b	96	7.5		
blk 1c	96	7.4		
blk 2a	96	8.1	Figure A.11 (starting with blk 2a in the top left, moving left to right down the page, ending with s 1c in the	74
blk 2b	96	7.7		
blk 2c	96	7.9		
s 1a	96	8.9		
s 1b	96	8.4		

(continued)

Table A.2. NO_3-N utilization experiment NO_3-N concentrations and corresponding chromatogram locations (continued).

Sample Code	Hour	NO_3-N(mg/L)	Chromatogram Appendix #	Appendix page #
s 1c	96	7.2	bottom right)	74
s 2a	96	8.3	Figure A.12 (starting with s 2a in the top left, moving left to right down the page, ending with s w TE 1c in the bottom right)	75
s 2b	96	8.9		
s 2c	96	8.8		
s w TE 1a	96	3.8		
s w TE 1b	96	3.6		
s w TE 1c	96	3.5		
s w TE 2a	96	2.6	Figure A.13 (starting with s w TE 2a in the top left, moving left to right down the page, ending with stk 1b in the bottom right)	76
s w TE 2b	96	2.5		
s w TE 2c	96	3.3		
7A	120	22.6		
stk 1a	120	7.9		
stk 1b	120	8.1		
stk 1c	120	8.0	Figure A.14 (starting with stk 1c in the top left, moving left to right down the page, ending with blk 1b in the bottom right)	77
stk 2a	120	8.8		
stk 2b	120	8.0		
stk 2c	120	9.1		
blk 1a	120	8.0		
blk 1b	120	8.2		
blk 1c	120	8.3	Figure A.15 (starting with blk 1c in the top left, moving left to right down the page, ending with s 1b in the bottom right)	78
blk 2a	120	8.0		
blk 2b	120	7.9		
blk 2c	120	7.7		
s 1a	120	5.6		
s 1b	120	10.3		
s 1c	120	10.2	Figure A.16 (starting with s 1c in the top left, moving left to right down the page, ending with s w TE 1b in the bottom right)	79
s 2a	120	7.0		
s 2b	120	8.1		
s 2c	120	8.7		
s w TE 1a	120	0.2		
s w TE 1b	120	3.3		
s w TE 1c	120	1.8	Figure A.17 (starting with s w TE 1c in the top left, moving left to right down the page,	80
s w TE 2a	120	2.2		

(continued)

Table A.2. NO_3-N utilization experiment NO_3-N concentrations and corresponding chromatogram locations (continued).

Sample Code	Hour	NO_3-N(mg/L)	Chromatogram Appendix #	Appendix page #
s w TE 2b	120	1.8	ending with stk 1a	80
s w TE 2c	120	1.6		
7A	144	22.6		
stk 1a	144	8.2		
stk 1b	144	8.1	Figure A.18 (starting with stk 1b in the top left, moving left to right down the page, ending with blk 1a in the bottom right)	81
stk 1c	144	8.3		
stk 2a	144	8.6		
stk 2b	144	8.5		
stk 2c	144	8.6		
blk 1a	144	7.8		
blk 1b	144	7.7	Figure A.19 (starting with blk 1b in the top left, moving left to right down the page, ending with s 1a in the bottom right)	82
blk 1c	144	7.8		
blk 2a	144	8.0		
blk 2b	144	8.5		
blk 2c	144	8.0		
s 1a	144	9.1		
s 1b	144	9.5	Figure A.20 (starting with s 1b in the top left, moving left to right down the page, ending with s w TE 1a in the bottom right)	83
s 1c	144	10.9		
s 2a	144	9.1		
s 2b	144	9.3		
s 2c	144	9.2		
s w TE 1a	144	0.3		
s w TE 1b	144	0.5	Figure A.21 (starting with s w TE 1b in the top left, moving left to right down the page, ending with R 1 in the bottom right)	84
s w TE 1c	144	0.2		
s w TE 2a	144	2.0		
s w TE 2b	144	1.9		
s w TE 2c	144	1.8		
R 1	144	8.0		
R 2	144	8.1	Figure A.22 (starting with R 2 in the top left, moving left to right down the page, ending with stk 1c in the bottom right)	85
R 3	144	7.8		
7A	168	22.6		
stk 1a	168	8.0		
stk 1b	168	8.1		
stk 1c	168	8.3		

(continued)

Table A.2. NO_3-N utilization experiment NO_3-N concentrations and corresponding chromatogram locations (continued).

Sample Code	Hour	NO_3-N(mg/L)	Chromatogram Appendix #	Appendix page #
stk 2a	168	8.3	Figure A.23 (starting with stk 2a in the top left, moving left to right down the page, ending with blk 1c in the bottom right)	86
stk 2b	168	8.0		
stk 2c	168	8.3		
blk 1a	168	8.3		
blk 1b	168	8.2		
blk 1c	168	8.3		
blk 2a	168	7.3	Figure A.24 (starting with blk 2a in the top left, moving left to right down the page, ending with s 1c in the bottom right)	87
blk 2b	168	8.0		
blk 2c	168	8.1		
s 1a	168	9.8		
s 1b	168	11.1		
s 1c	168	9.2		
s 2a	168	8.5	Figure A.25 (starting with s 2a in the top left, moving left to right down the page, ending with s w TE 1c in the bottom right)	88
s 2b	168	9.0		
s 2c	168	8.4		
s w TE 1a	168	0.3		
s w TE 1b	168	0.2		
s w TE 1c	168	0.3		
s w TE 2a	168	2.4	Figure A.26 (starting with s w TE 2a in the top left, moving left to right down the page, ending with R 3 in the bottom right)	89
s w TE 2b	168	2.4		
s w TE 2c	168	2.0		
R 1	168	7.6		
R 2	168	7.9		
R 3	168	7.7		

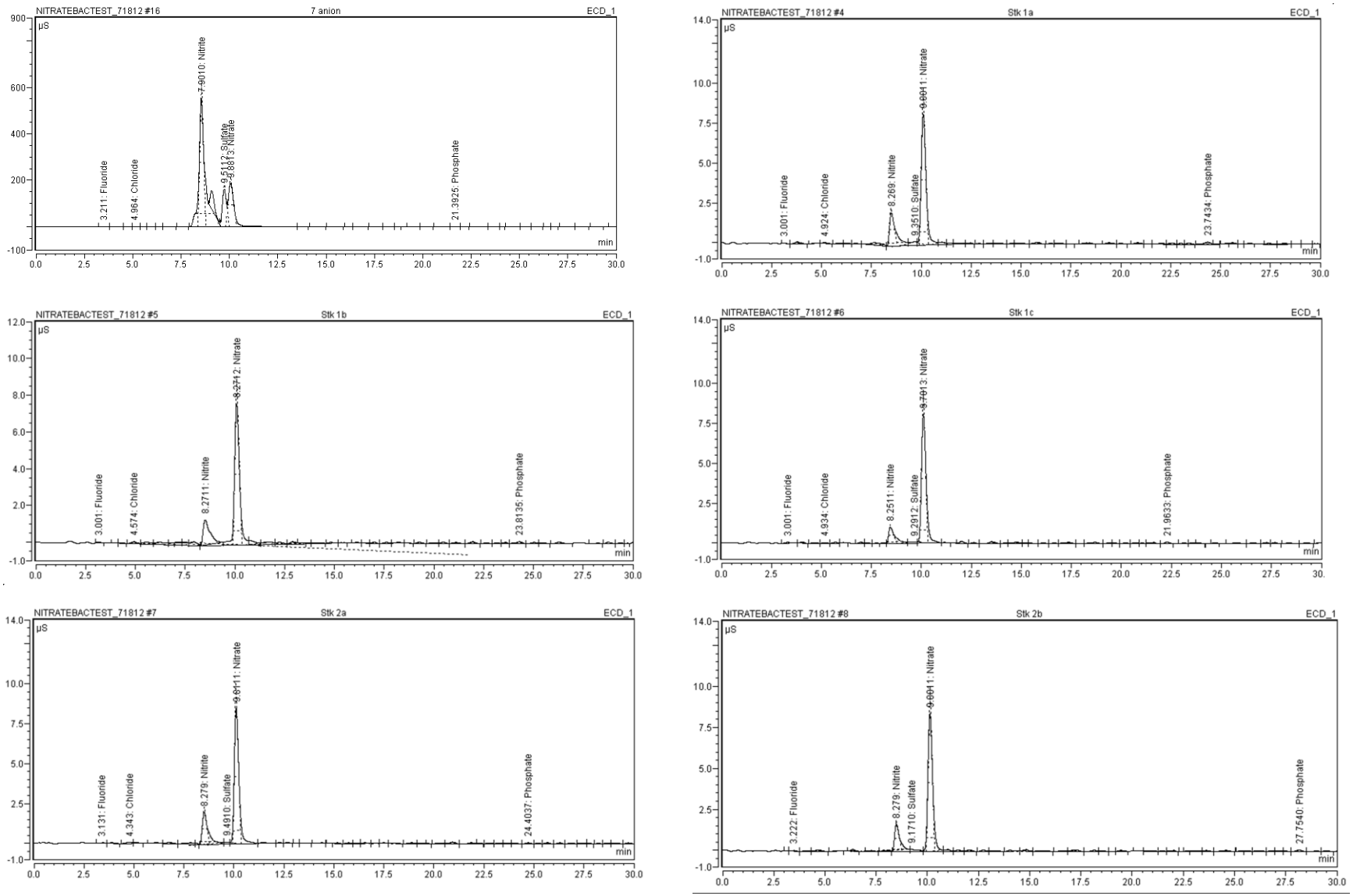


Figure A.1. Chromatograms of 7A, stk 1a, stk 1b, stk 1c, stk 2a, and stk 2b after 48 hours.

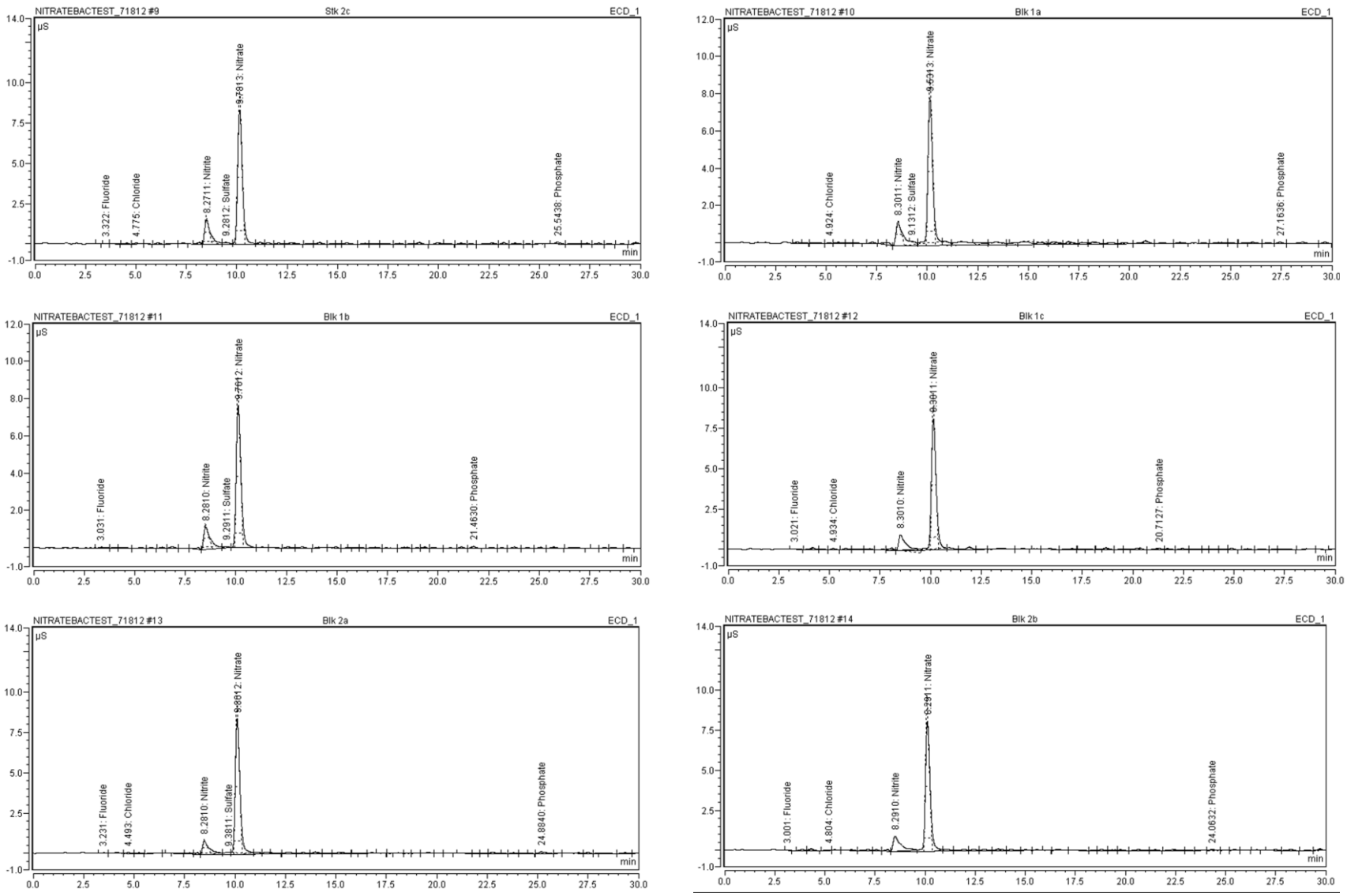


Figure A.2. Chromatograms of stk 2c, blk 1a, blk 1b, blk 1c, blk 2a, and blk 2b after 48 hours.

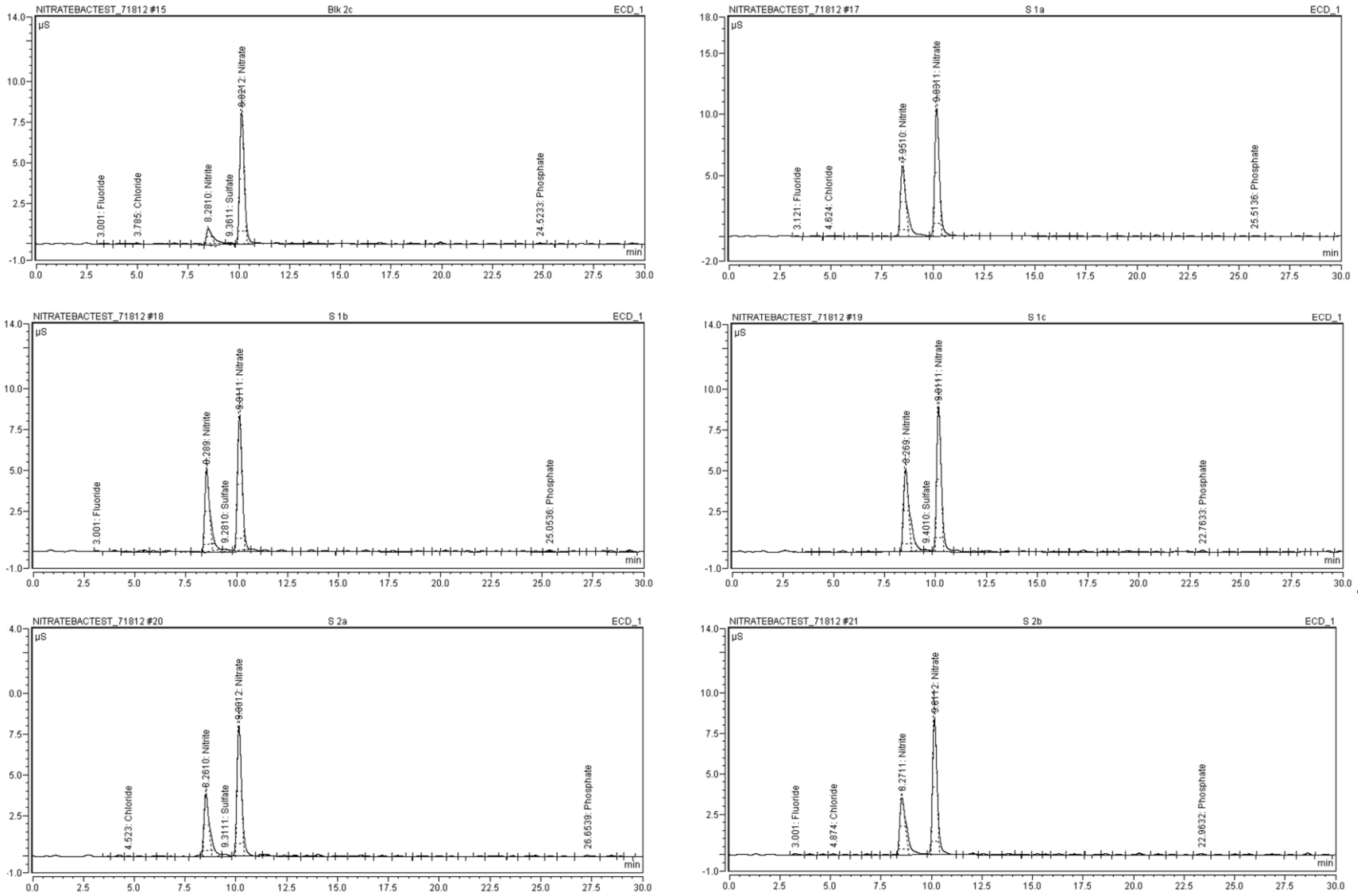


Figure A.3. Chromatograms of blk 2c, s 1a, s 1b, s 1c, s 2a, and s 2b after 48 hours.

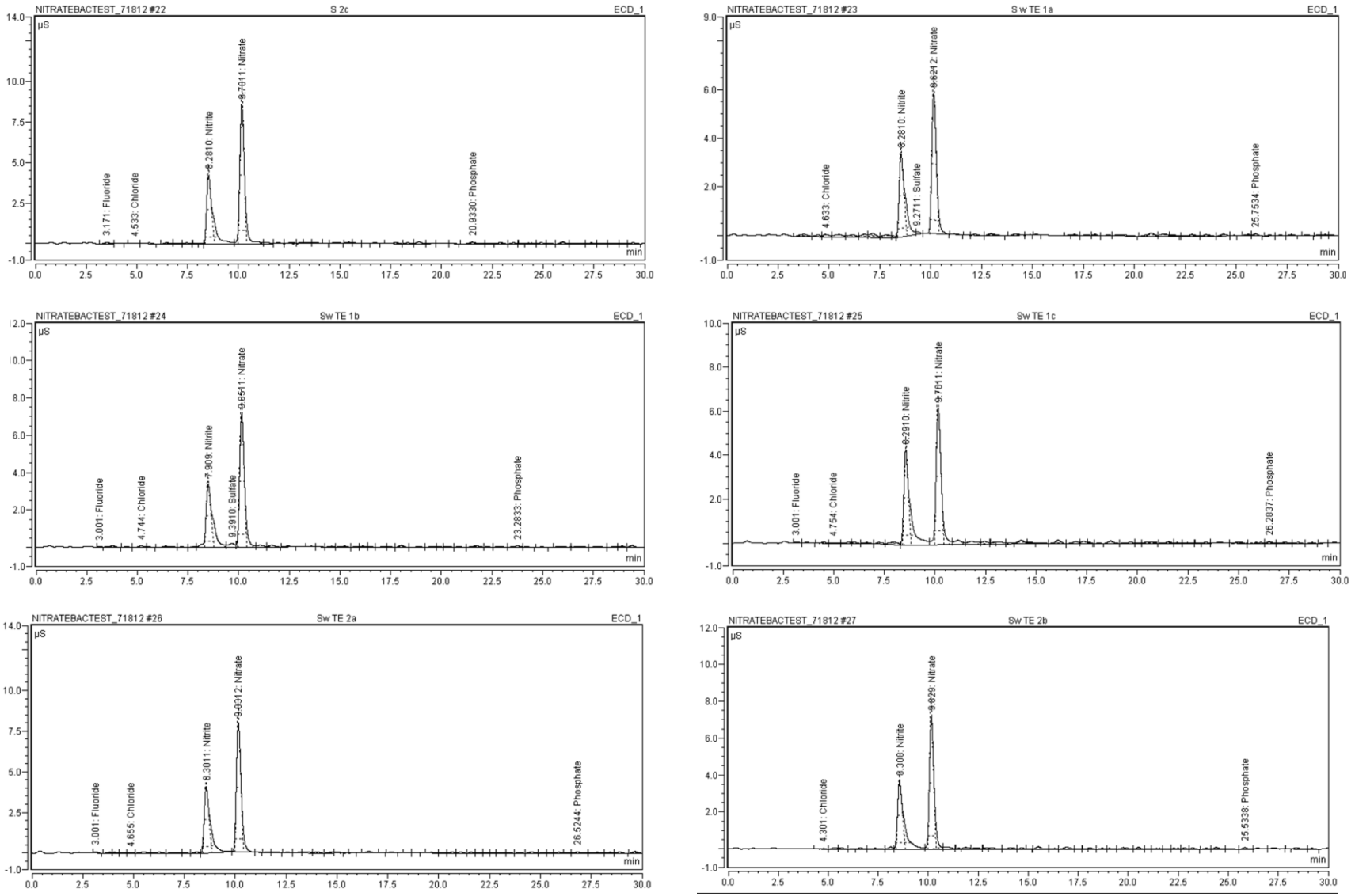


Figure A.4. Chromatograms of s 2c, s w TE 1a, s w TE 1b, s w TE 1c, s w TE 2a, and s w TE 2b after 48 hours.

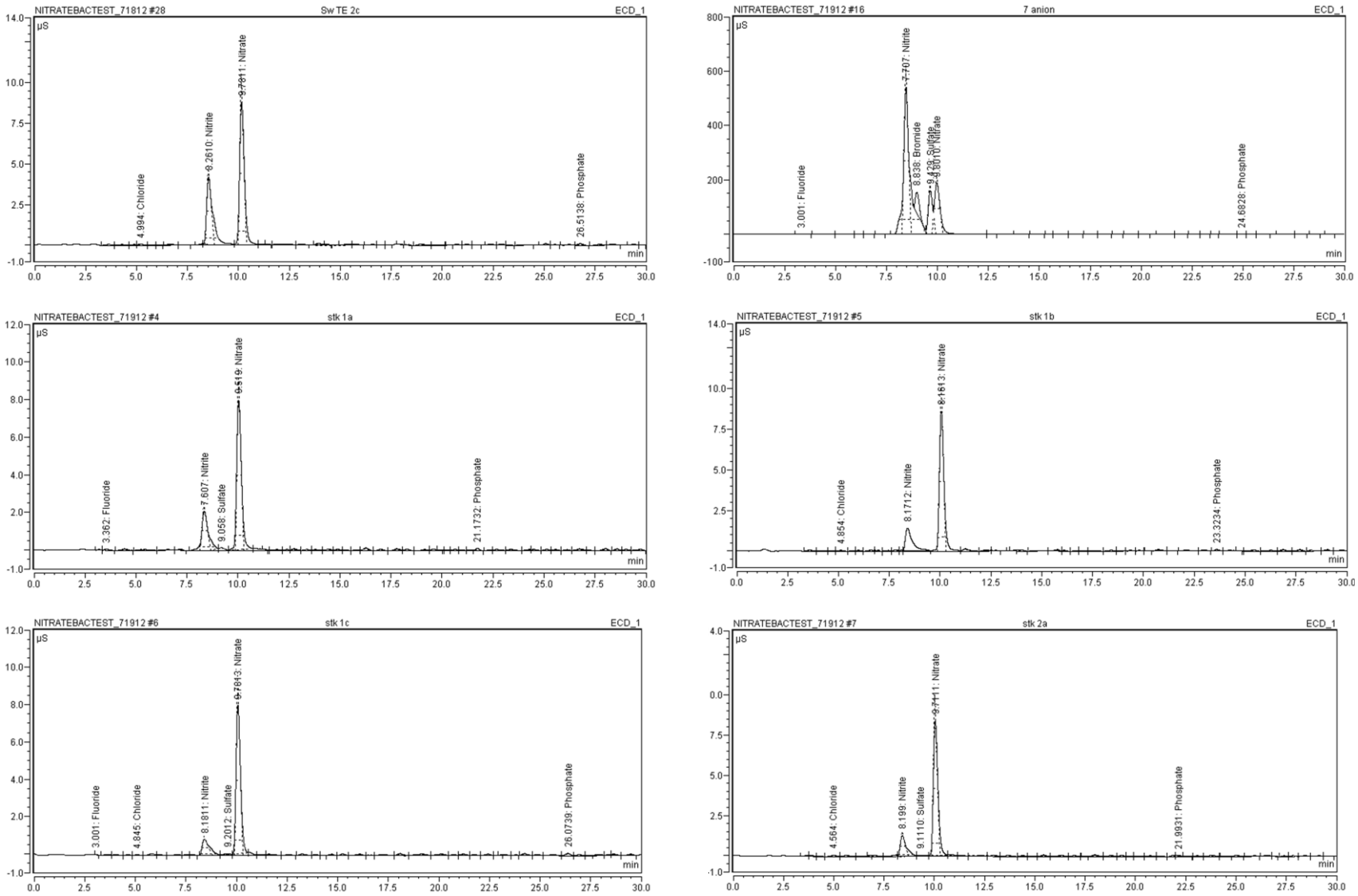


Figure A.5. Chromatograms of s w TE 2c (after 48 hours), 7A, stk 1a, stk 1b, stk 1c, and stk 2a after 72 hours.

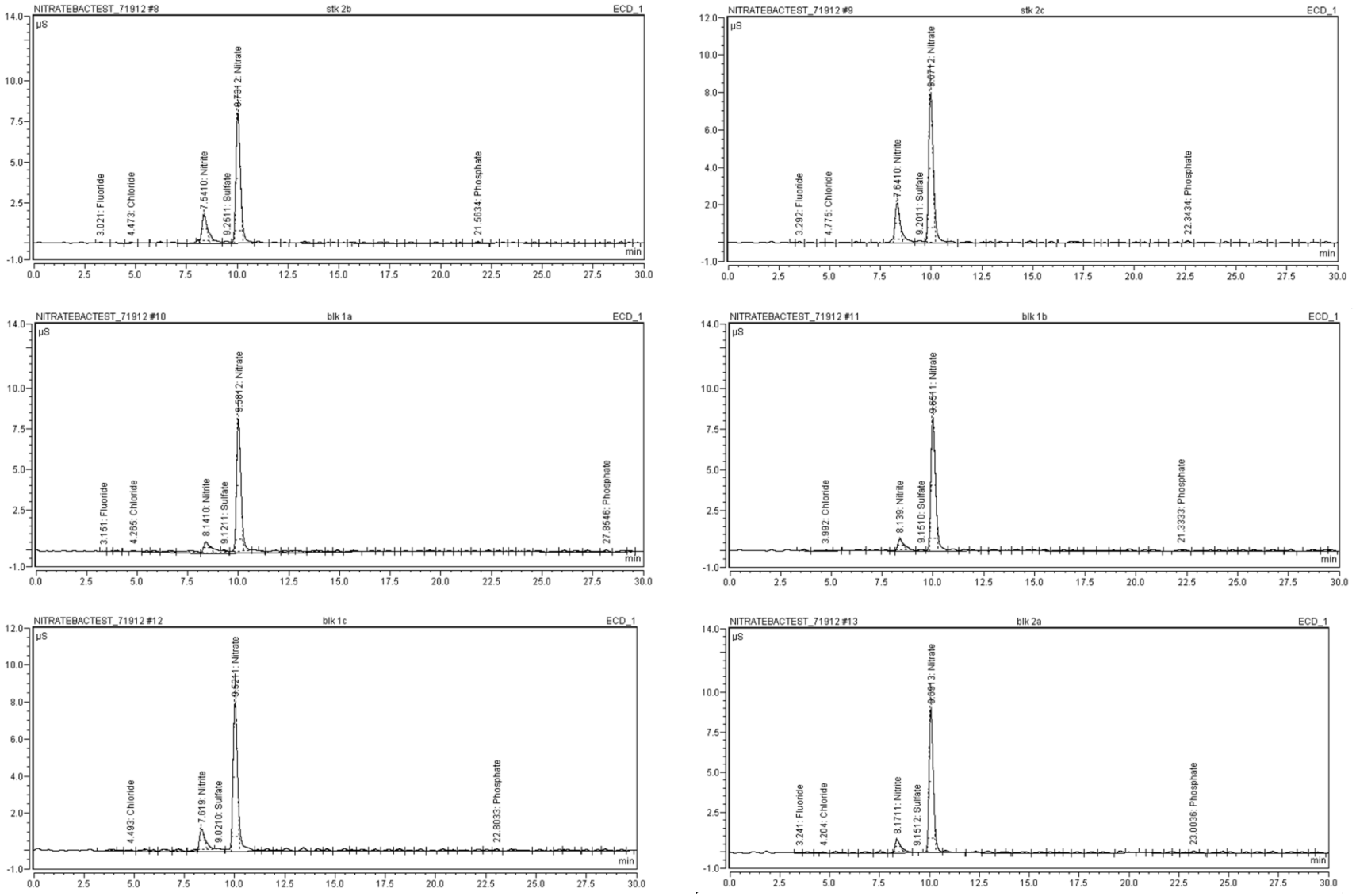


Figure A.6. Chromatograms of stk 2b, stk 2c, blk 1a, blk 1b, blk 1c, and blk 2a after 72 hours. .

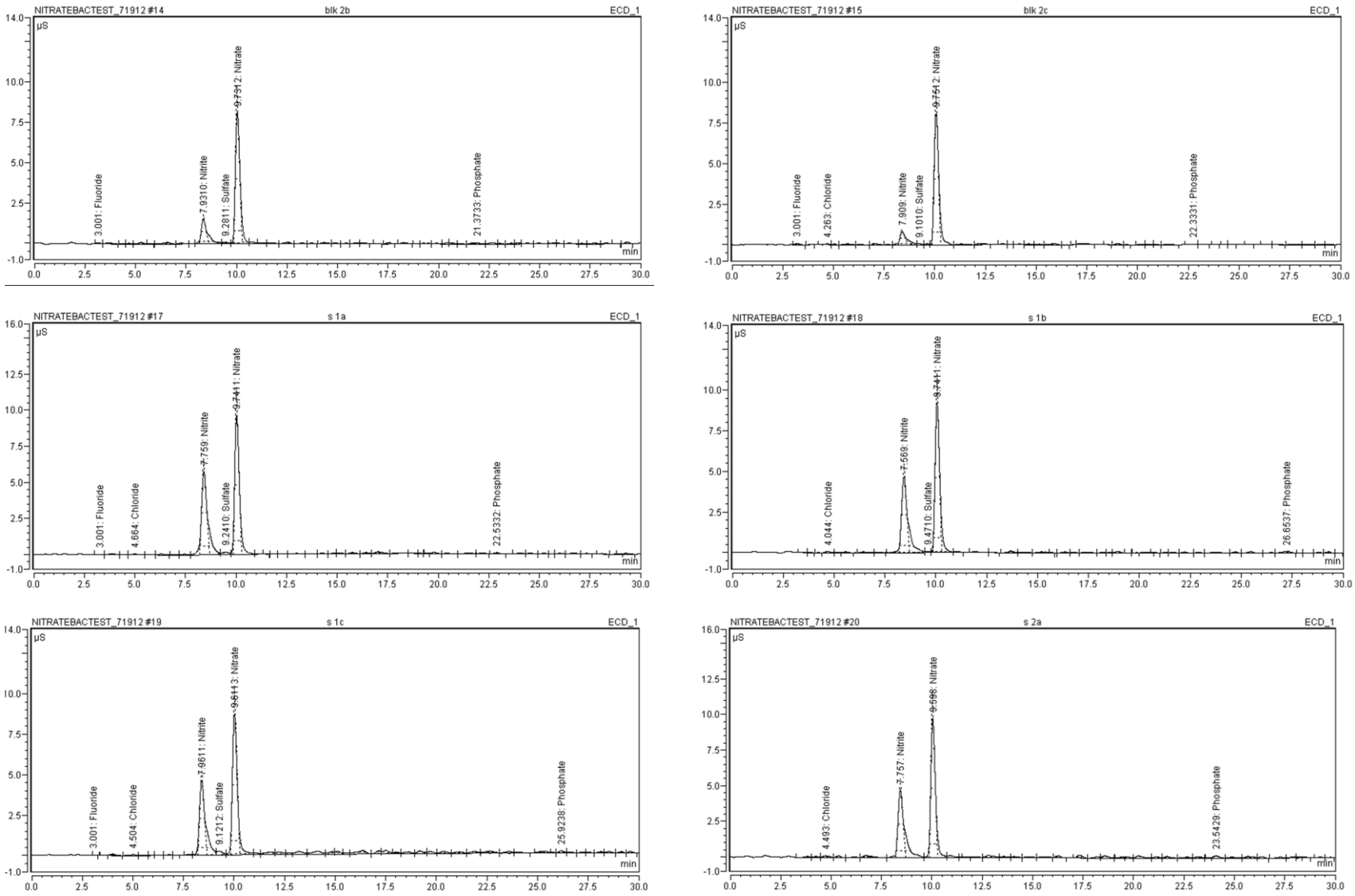


Figure A.7. Chromatograms of blk 2b, blk 2c, s 1a, s 1b, s 1c, and s 2a after 72 hours.

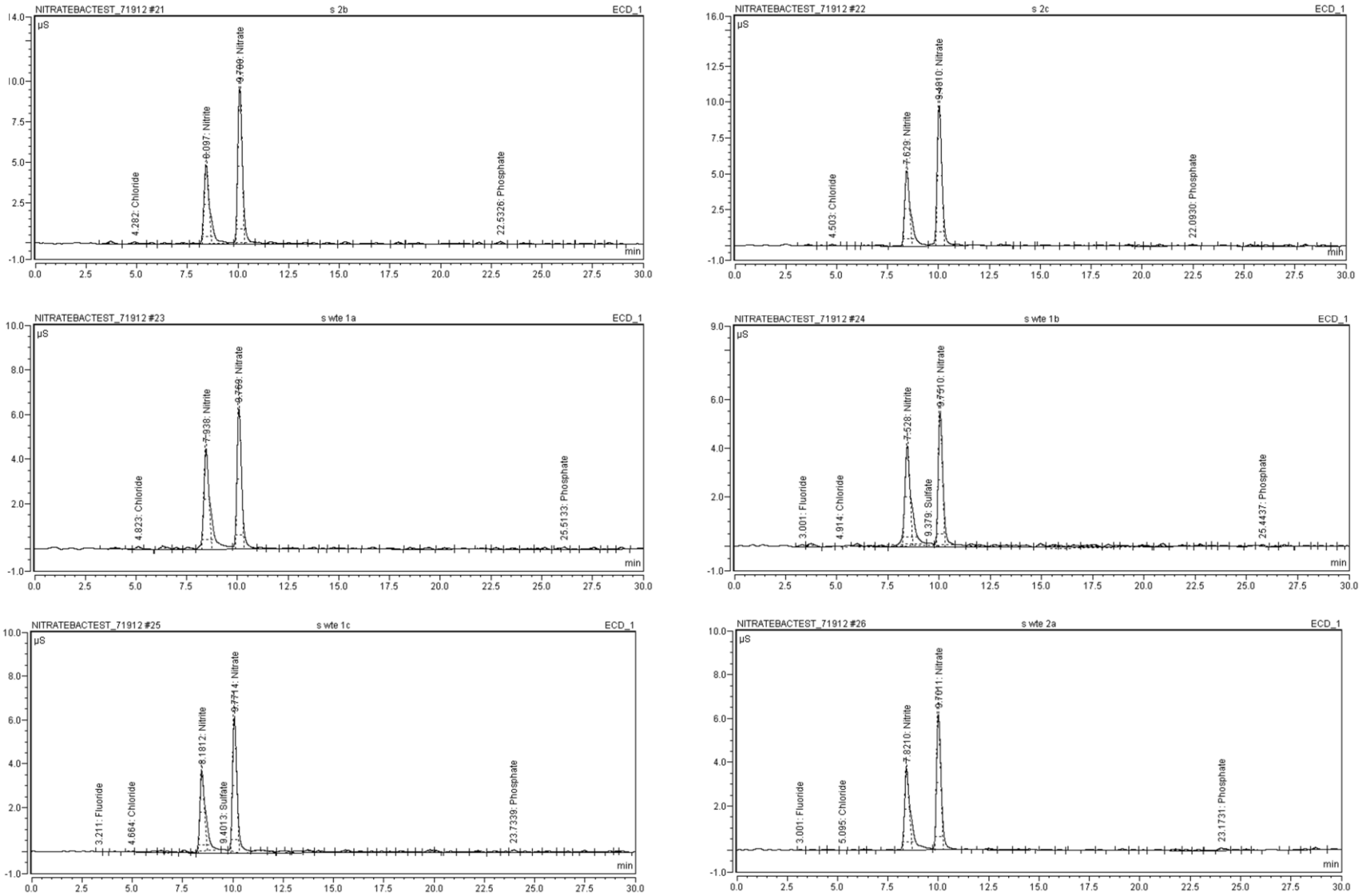


Figure A.8. Chromatograms of s 2b, s 2c, s w TE 1a, s w TE 1b, s w TE 1c, and s w TE 2a after 72 hours.

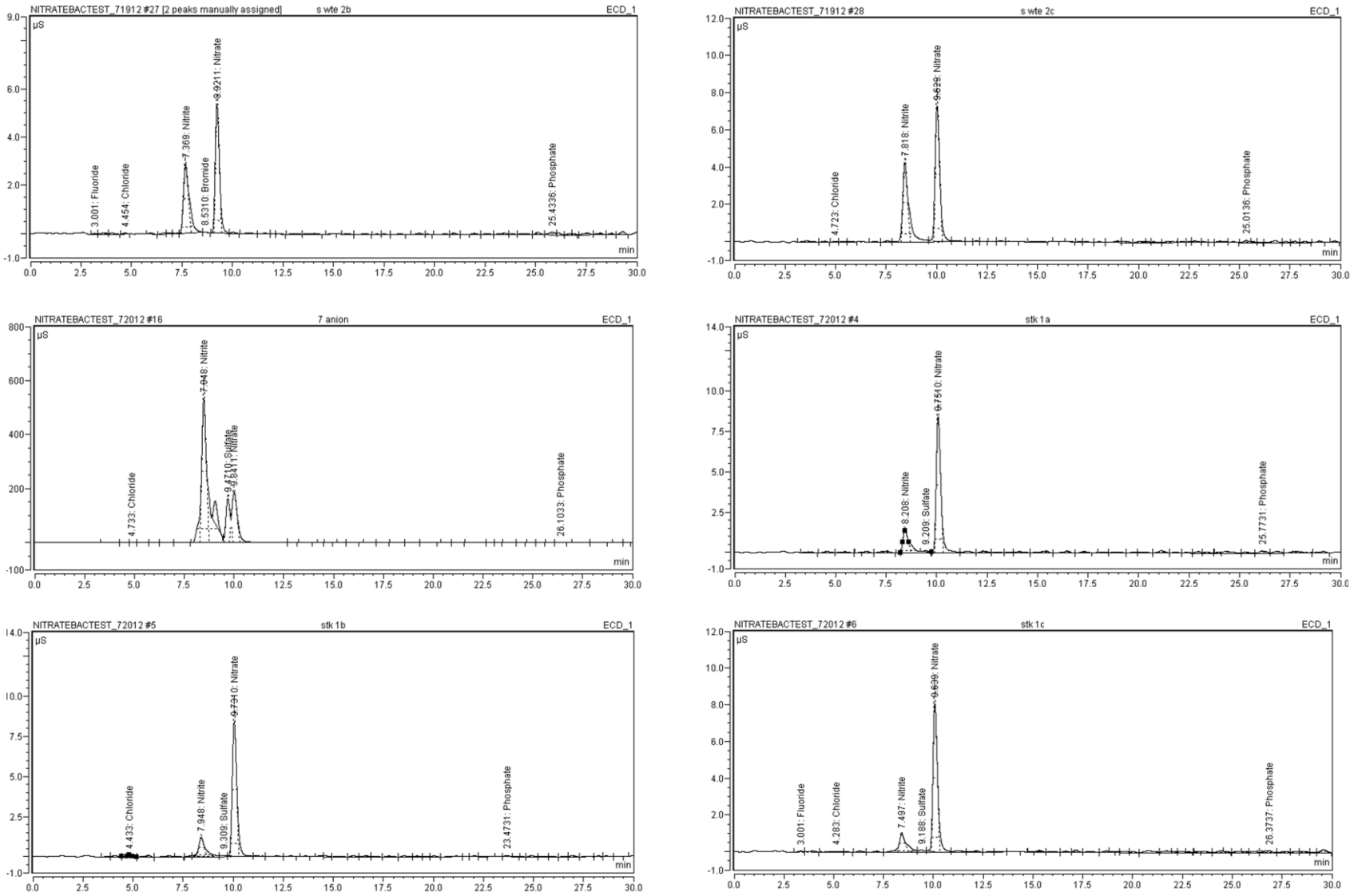


Figure A.9. Chromatograms of s w TE 2b (after 72 hours), s w TE 2c (after 72 hours), 7A, stk 1a, stk 1b, and stk 1c after 96 hours.

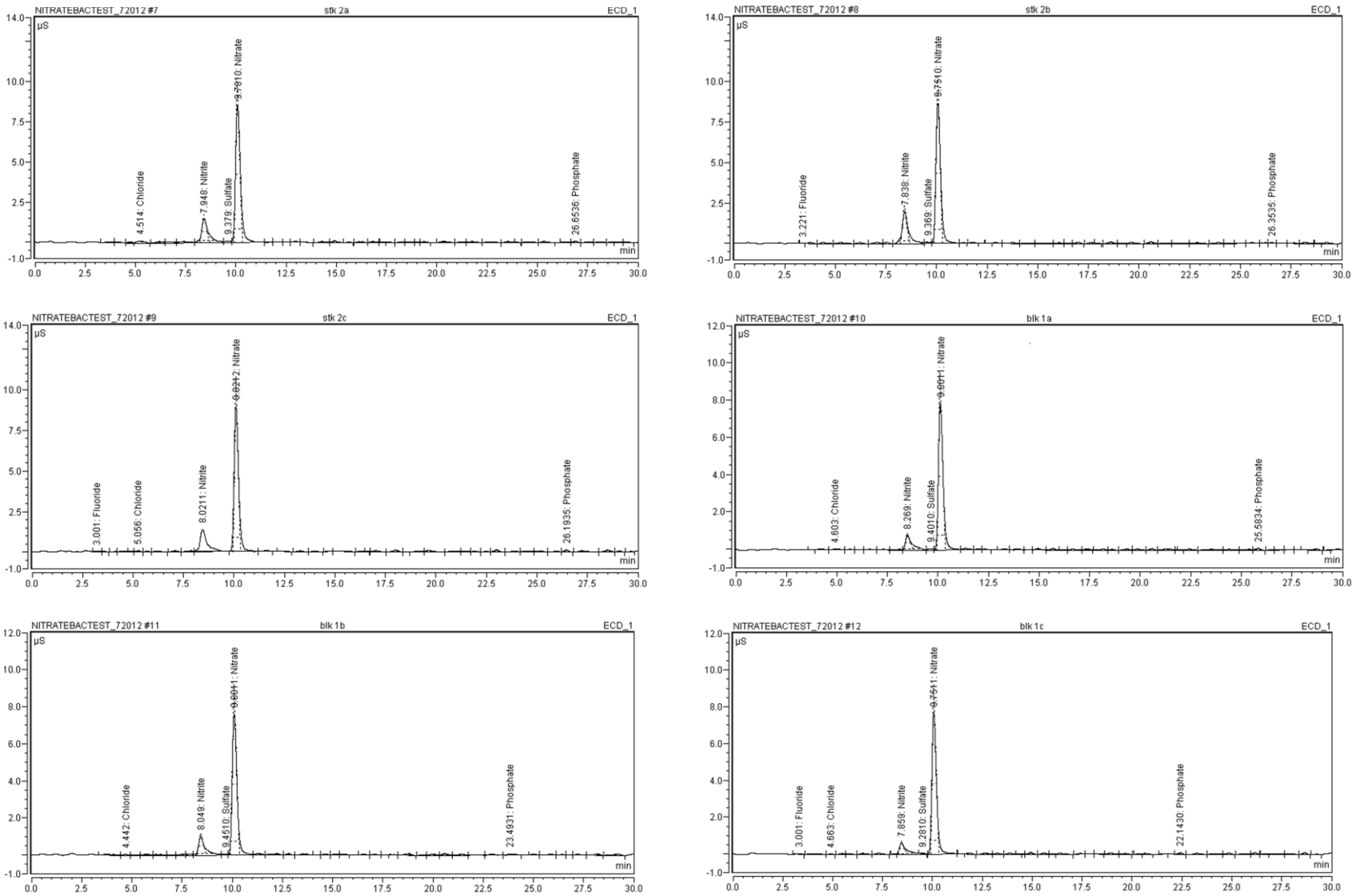


Figure A.10. Chromatograms of stk 2a, stk 2b, stk 2c, blk 1a, blk 1b, and blk 1c after 96 hours.

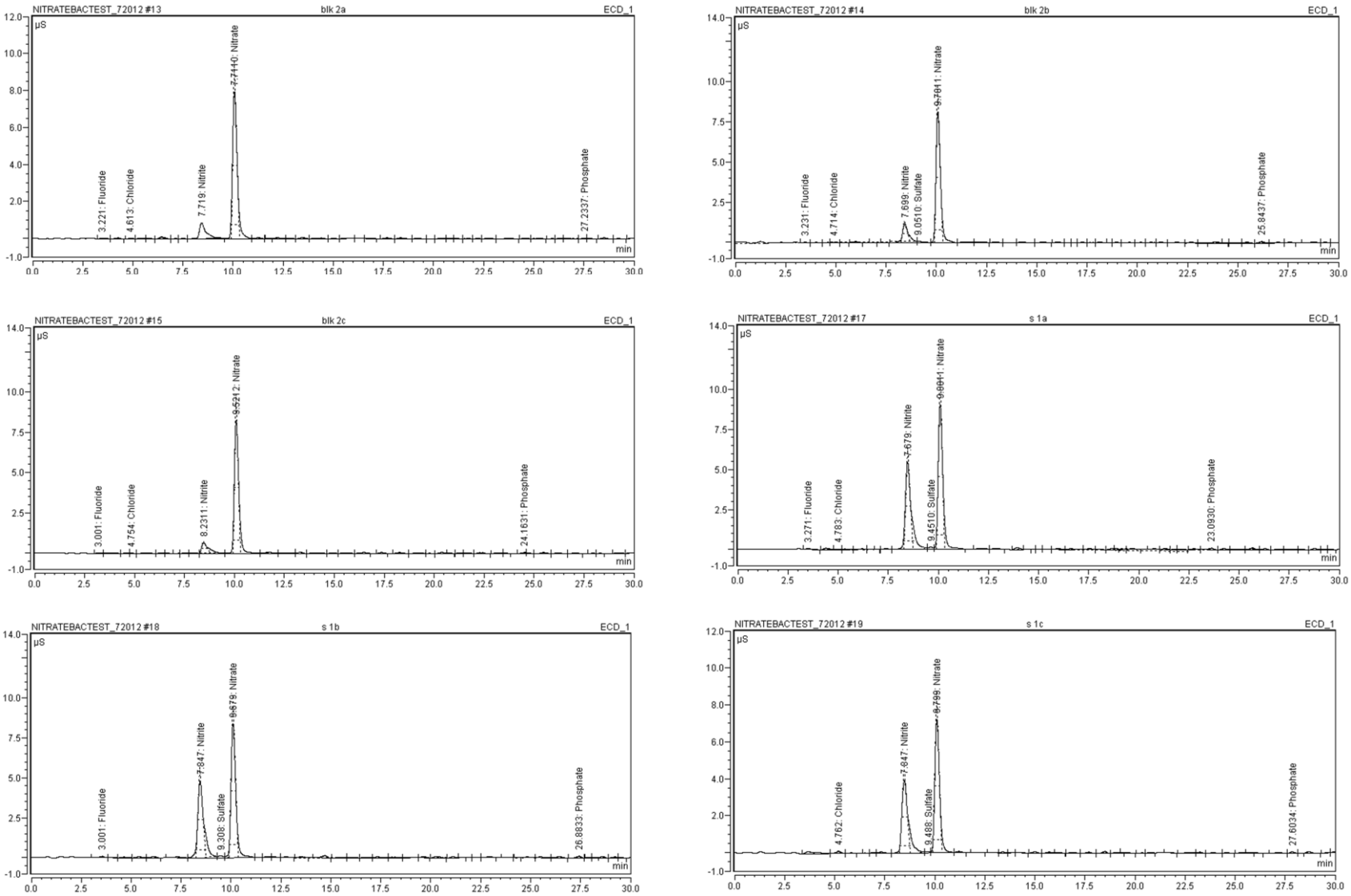


Figure A.11. Chromatograms of blk 2a, blk 2b, blk 2c, s 1a, s 1b, and s 1c after 96 hours.

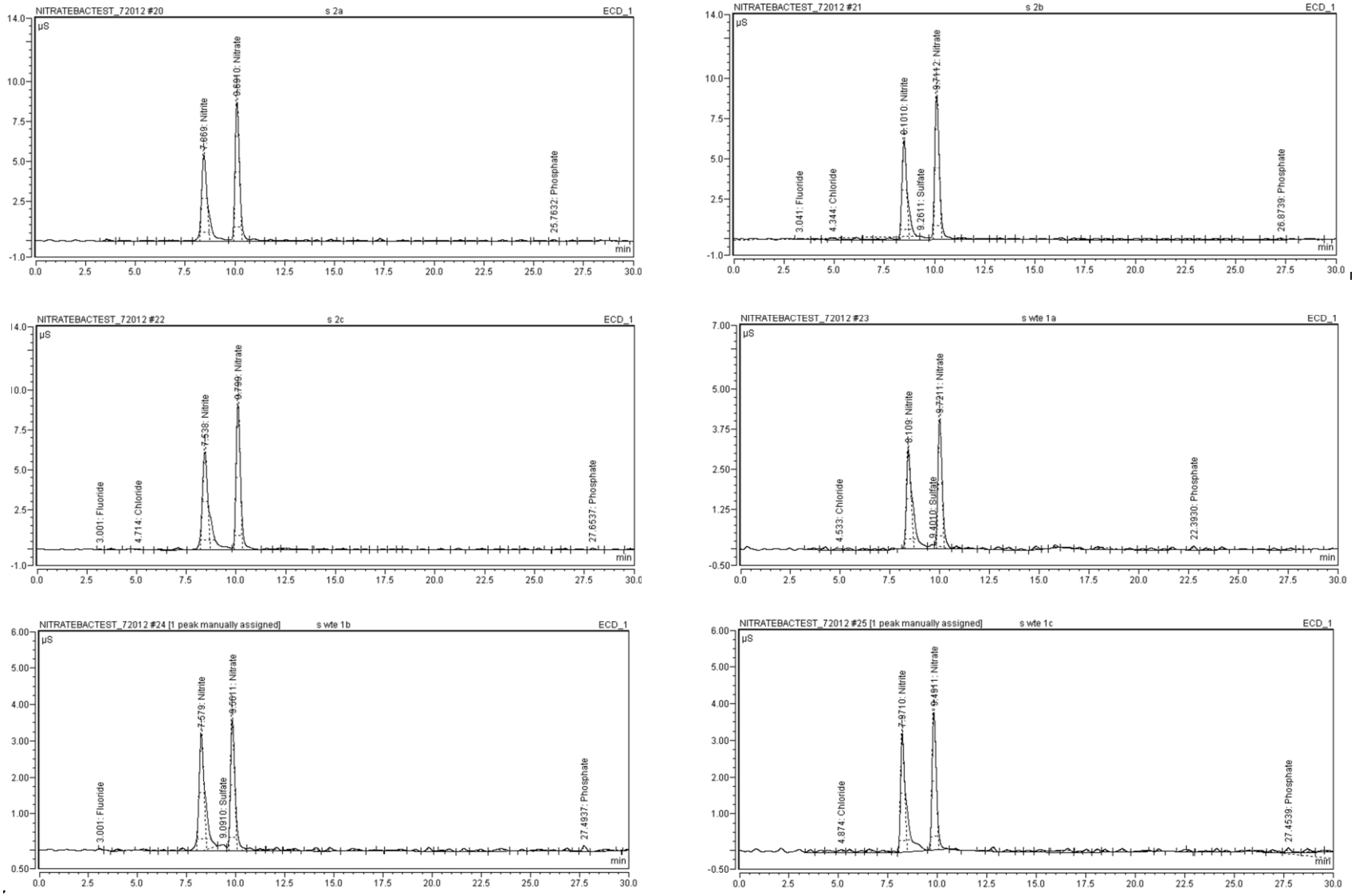


Figure A.12. Chromatograms of s 2a, s 2b, s 2c, s w TE 1a, s w TE 1b, and s w TE 1c after 96 hours.

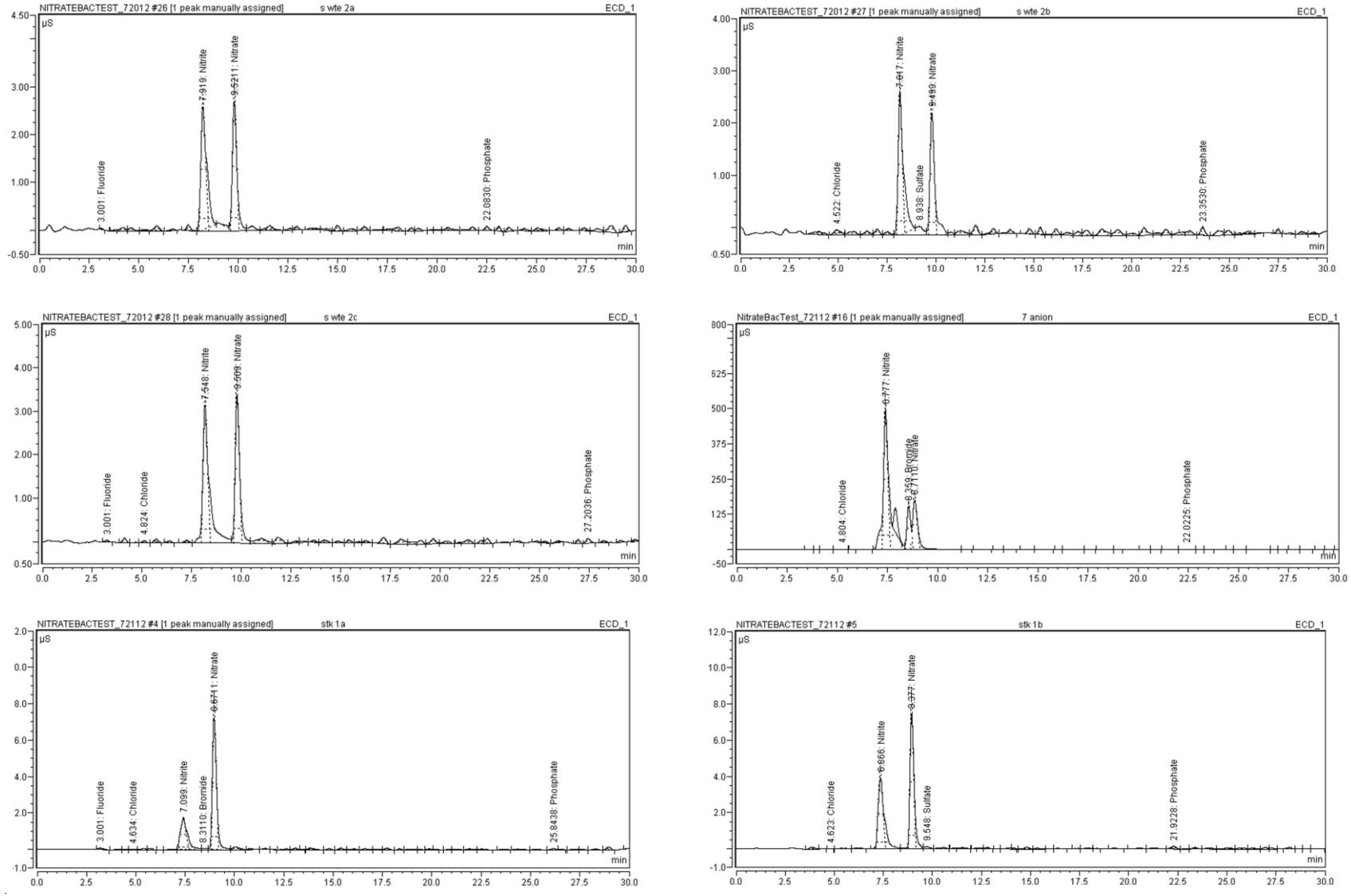


Figure A.13. Chromatograms of s w TE 2a (after 96 hours), s w TE 2b (after 96 hours), s w TE 2c (after 96 hours), 7A (after 120 hours), stk 1a (after 120 hours), and stk 1b (after 120 hours).

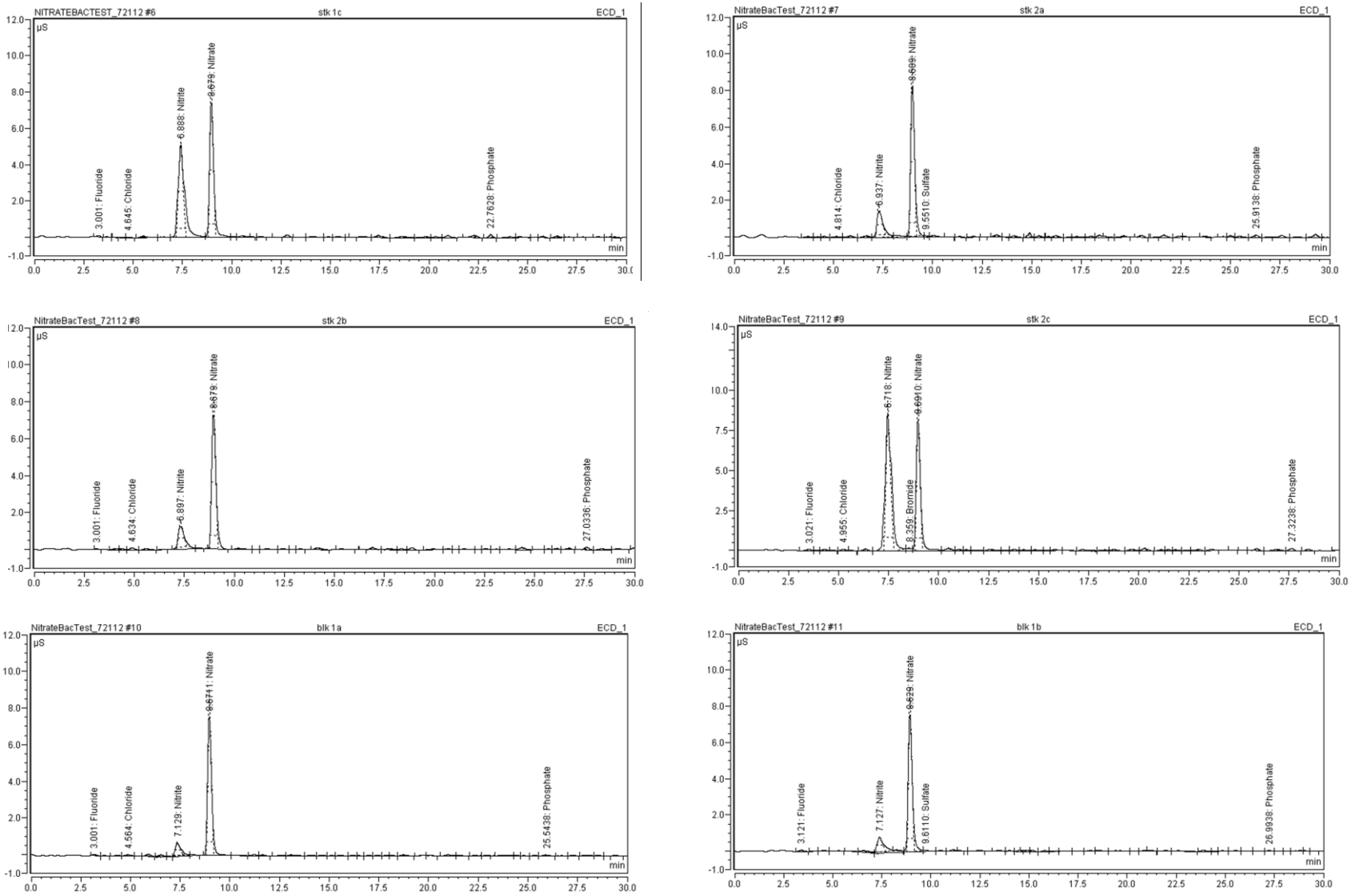


Figure A.14. Chromatograms of stk 1c, stk 2a, stk 2b, stk 2c, blk 1a, and blk 1b after 120 hours.

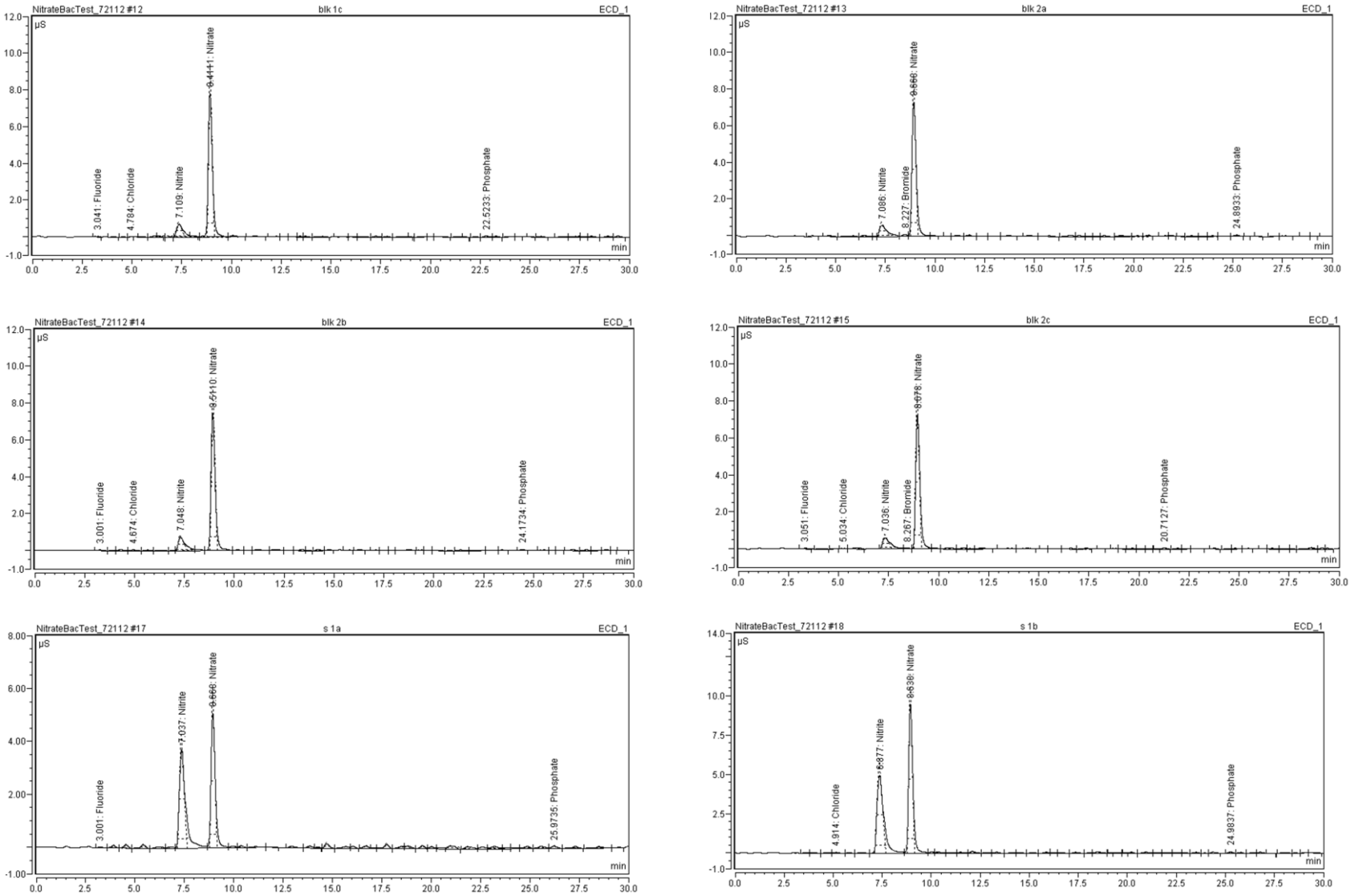


Figure A.15. Chromatograms of blk 1c, blk 2a, blk 2b, blk 2c, s 1a, and s 1b after 120 hours.

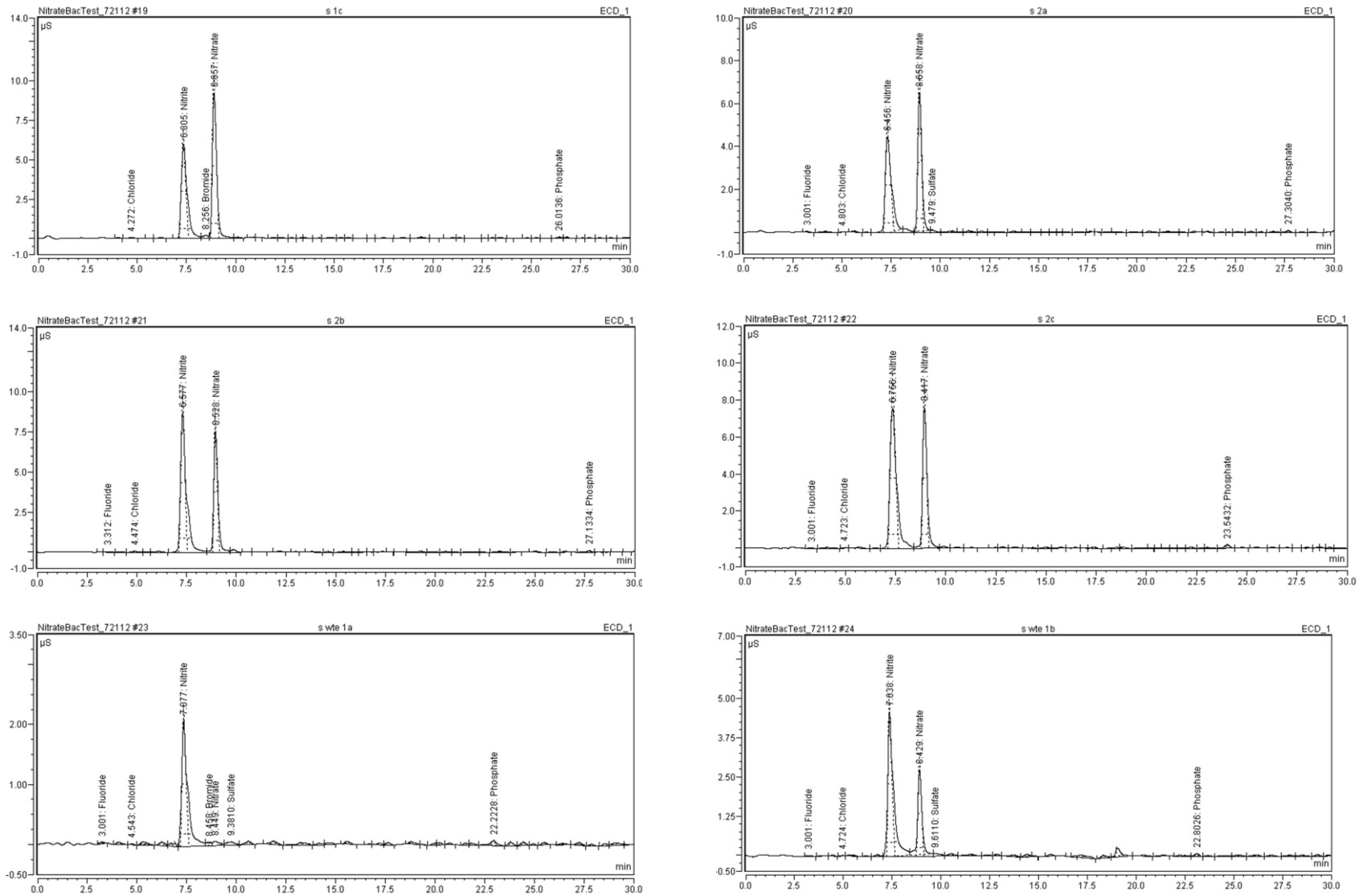


Figure A.16. Chromatograms of s 1c, s 2a, s 2b, s 2c, s w TE 1a, and s w TE 1b after 120 hours.

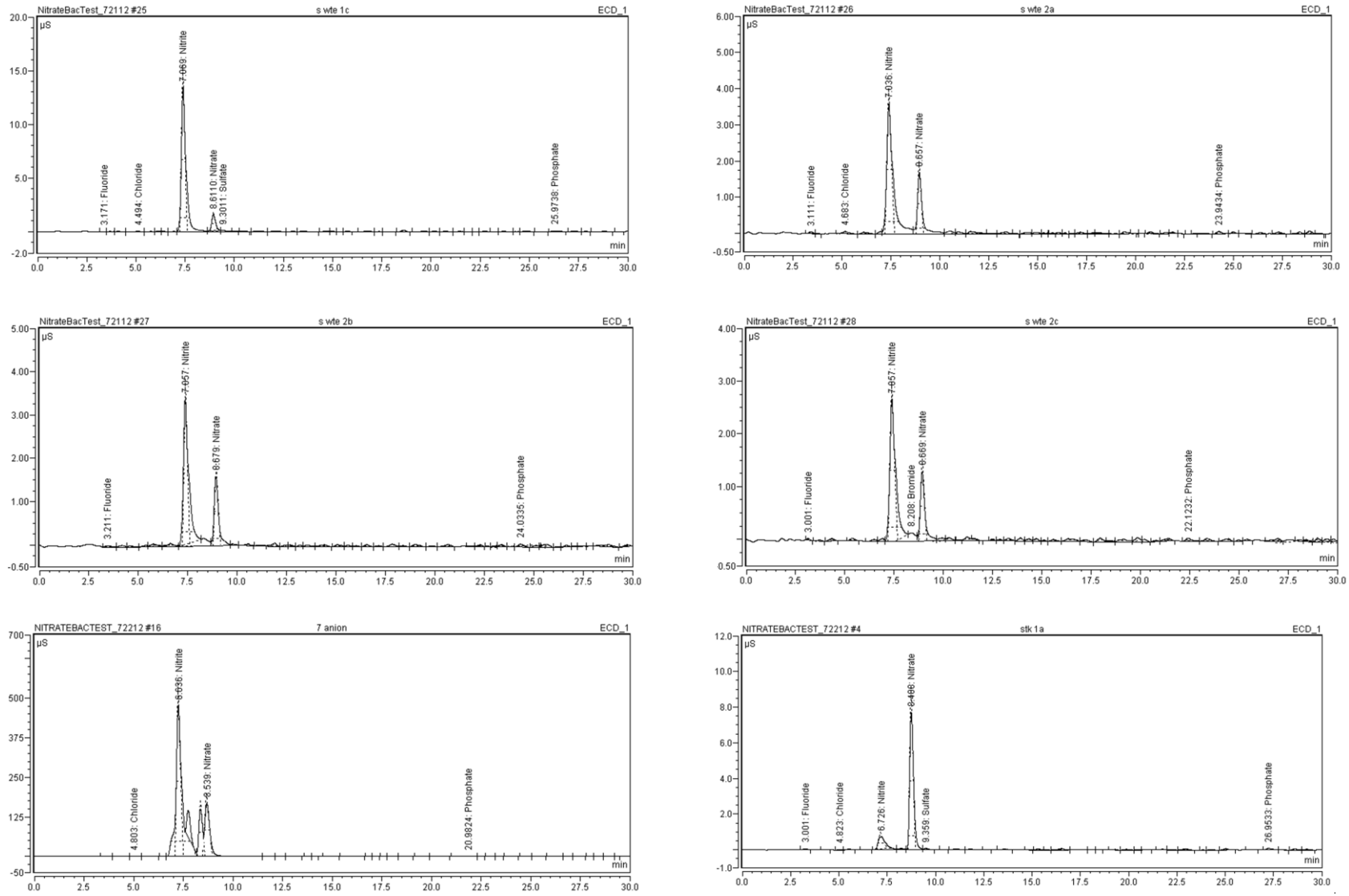


Figure A.17. Chromatograms of s w TE 1c (after 120 hours), s w TE 2a (after 120 hours), s w TE 2b (after 120 hours), s w TE 2c (after 120 hours), 7A (after 144 hours), and stk 1a (after 144 hours).

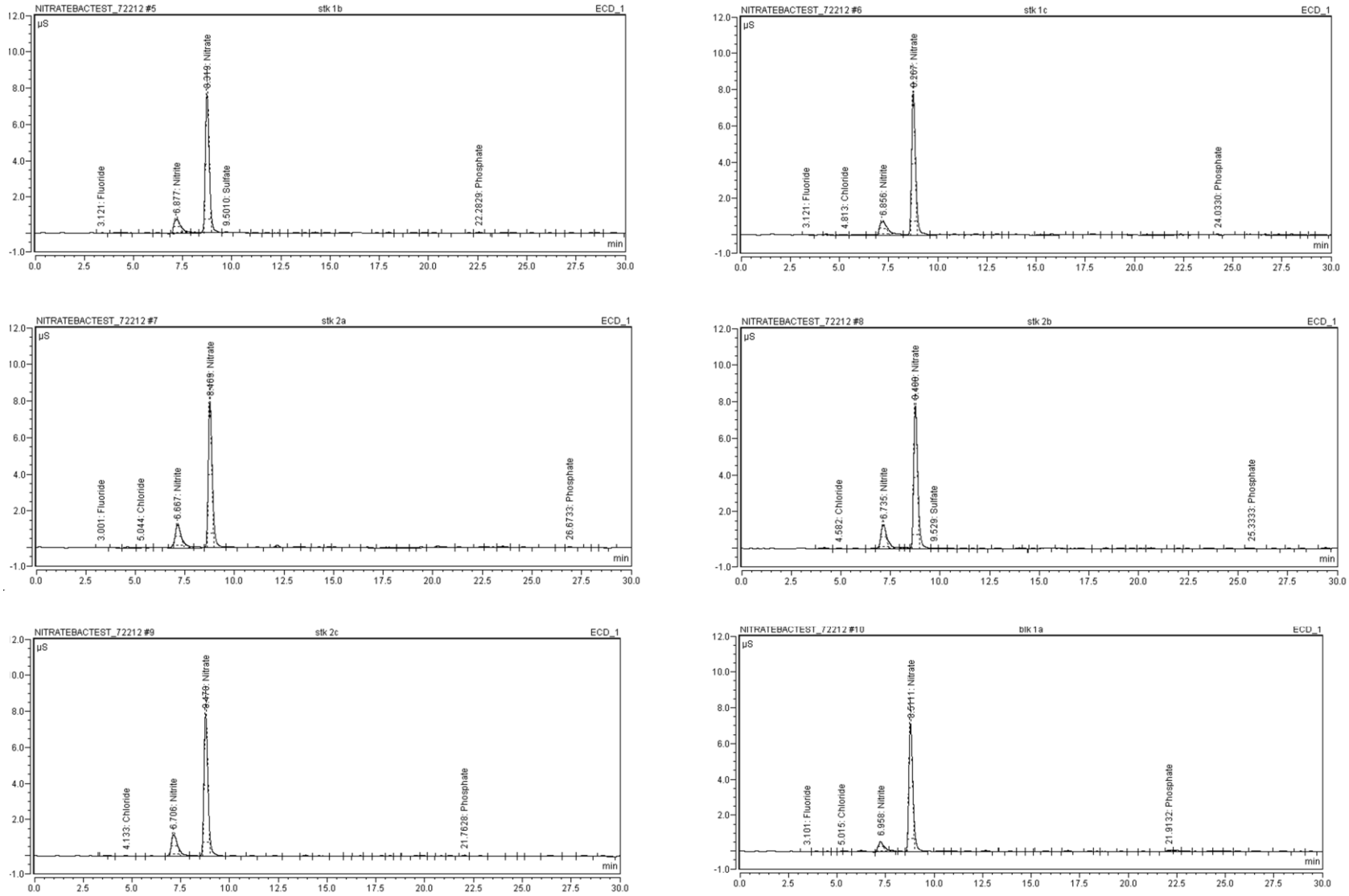


Figure A.18. Chromatograms of stk 1b, stk 1c, stk 2a, stk 2b, stk 2c, and blk 1a after 144 hours.

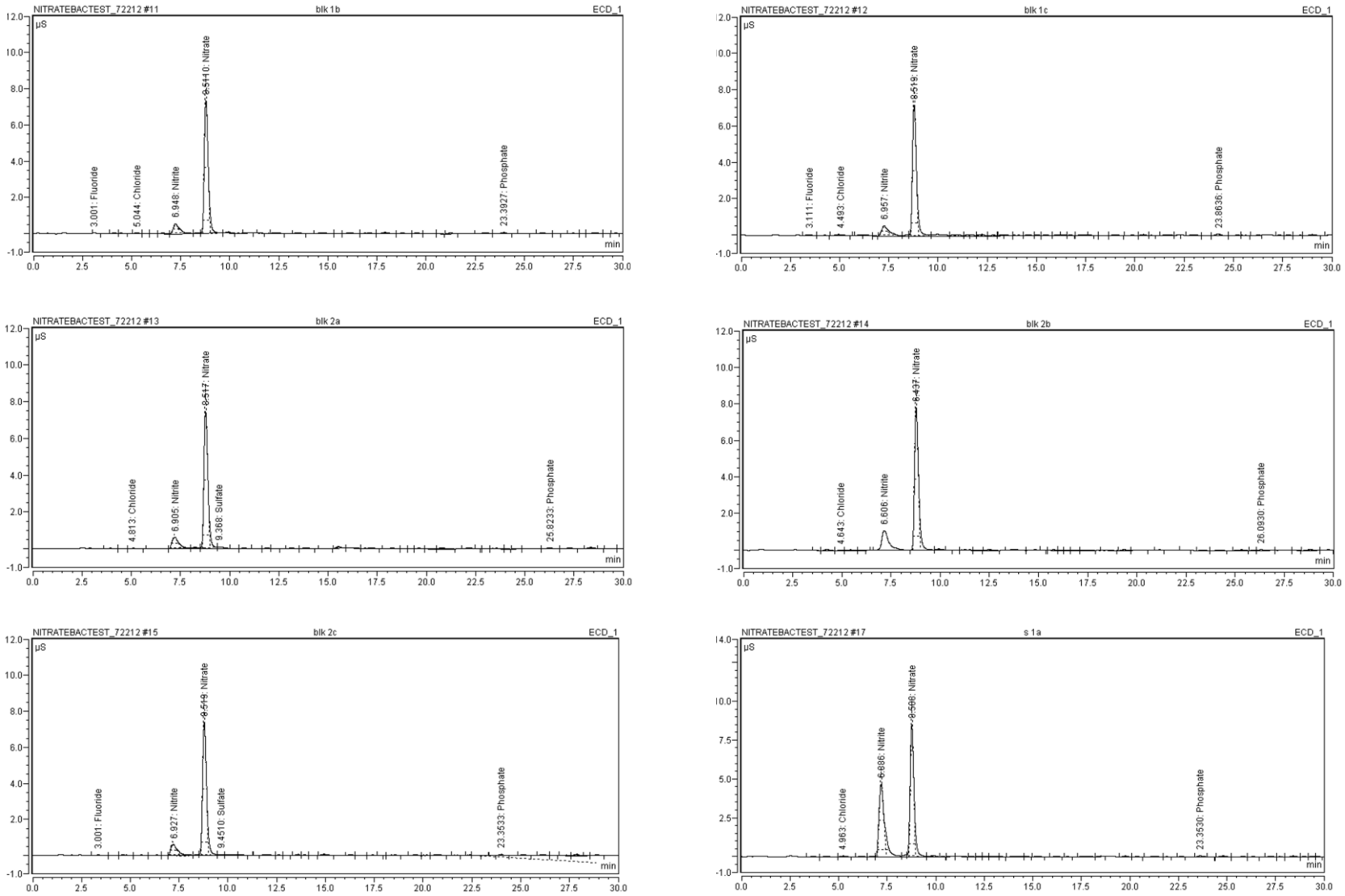


Figure A.19. Chromatograms of blk 1b, blk 1c, blk 2a, blk 2b, blk 2c, and s 1a after 144 hours.

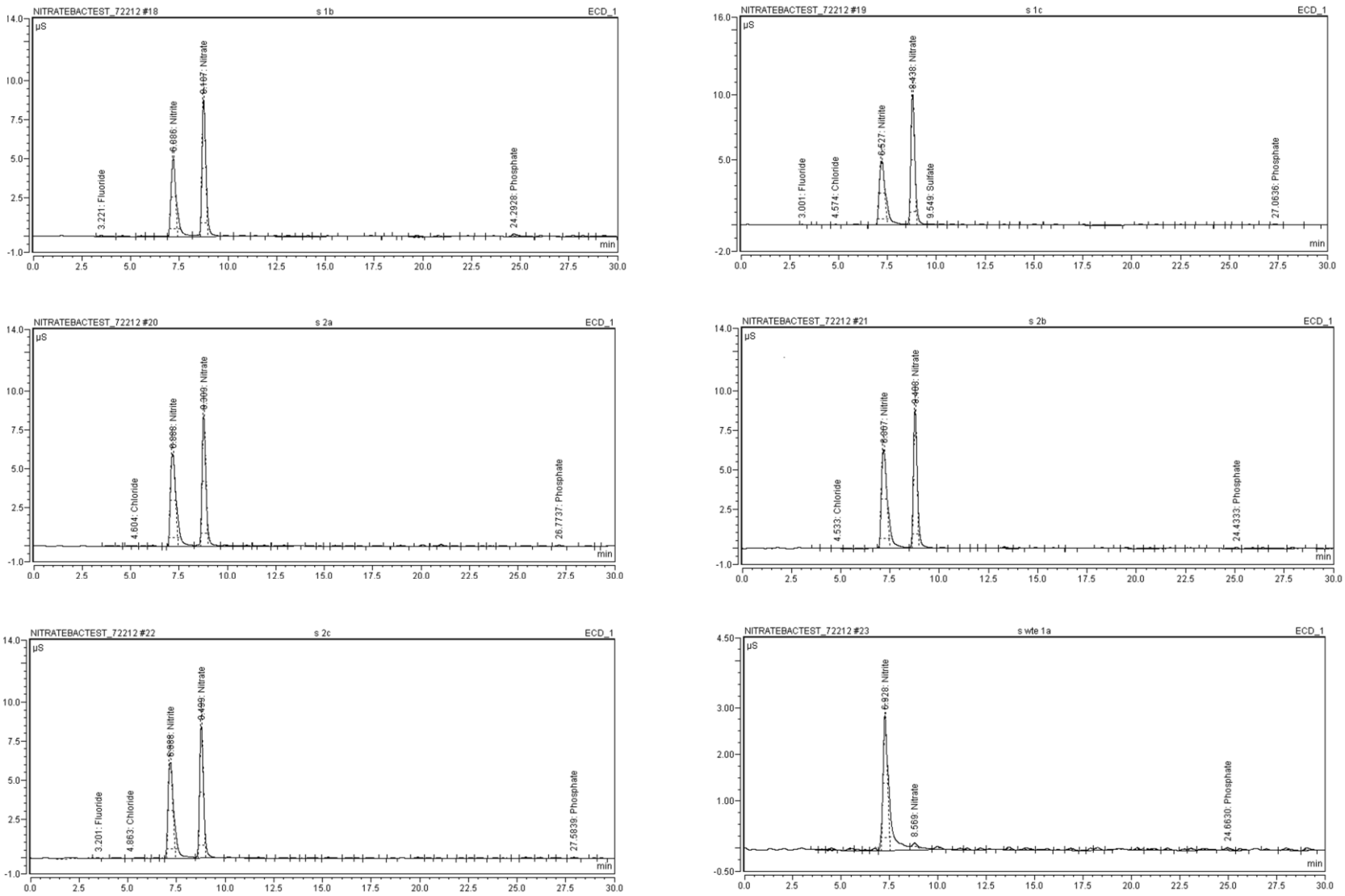


Figure A.20. Chromatograms of s 1b, s 1c, s 2a, s 2b, s 2c, and s w TE 1a after 144 hours.

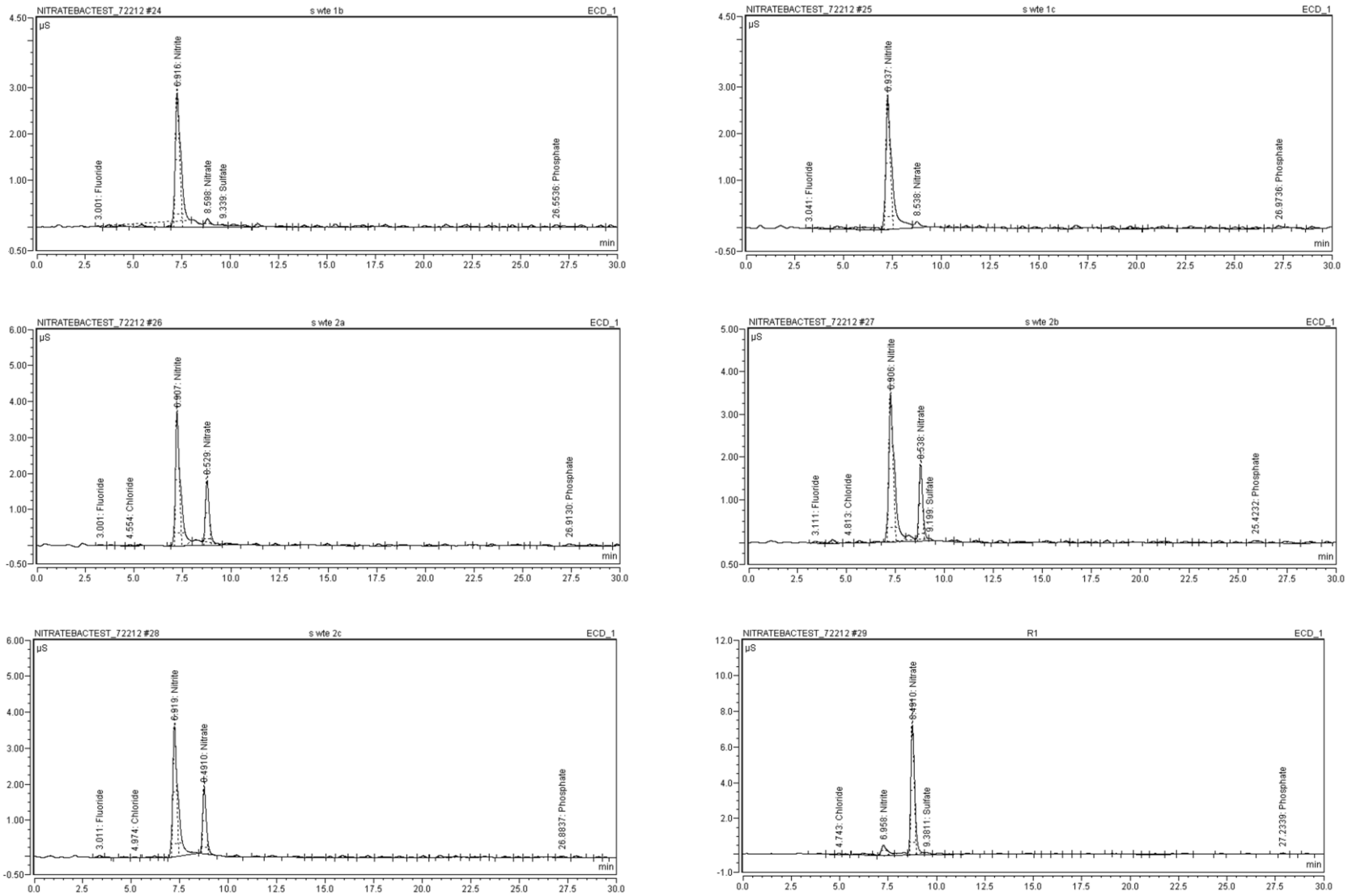


Figure A.21. Chromatograms of s w TE 1b, s w TE 1c, s w TE 2a, s w TE 2b, s w TE 2c, and R 1 after 144 hours.

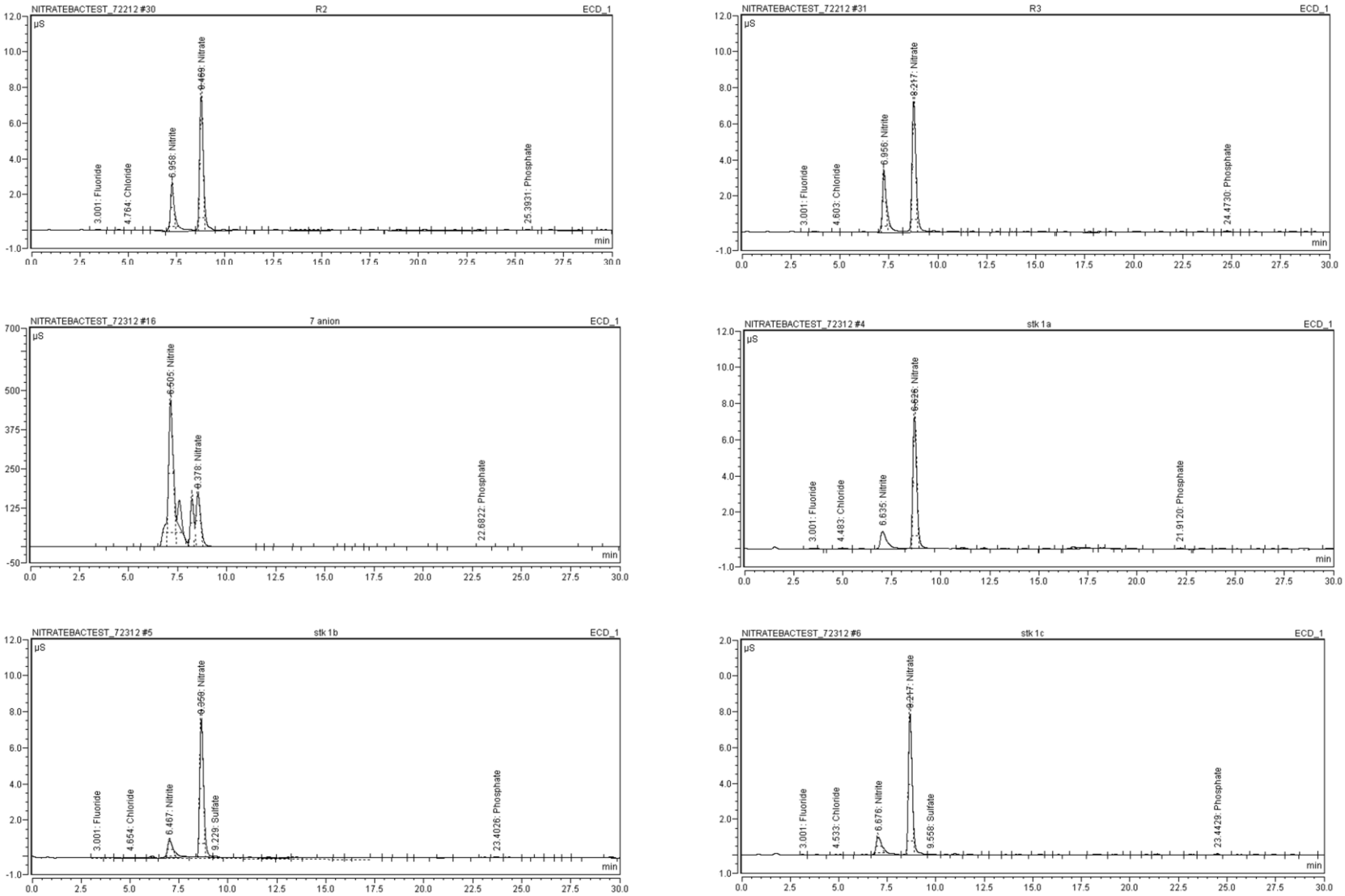


Figure A.22. Chromatograms of R 2 (after 144 hours), R 3 (after 144 hours), 7A, stk 1a, stk 1b, and stk 1c after 168 hours.

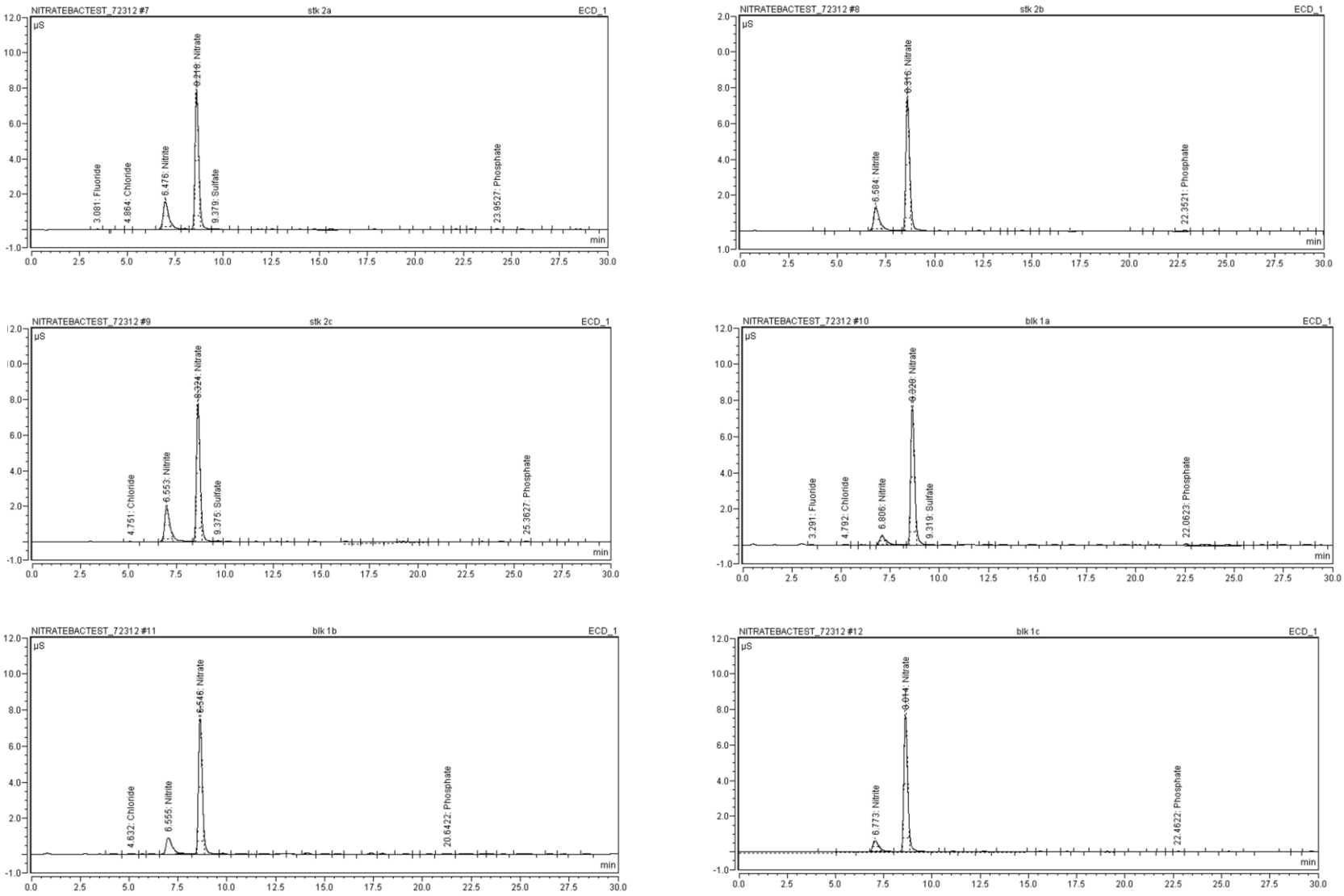


Figure A.23. Chromatograms of stk 2a, stk 2b, stk 2c, blk 1a, blk 1b, and blk 1c after 168 hours.

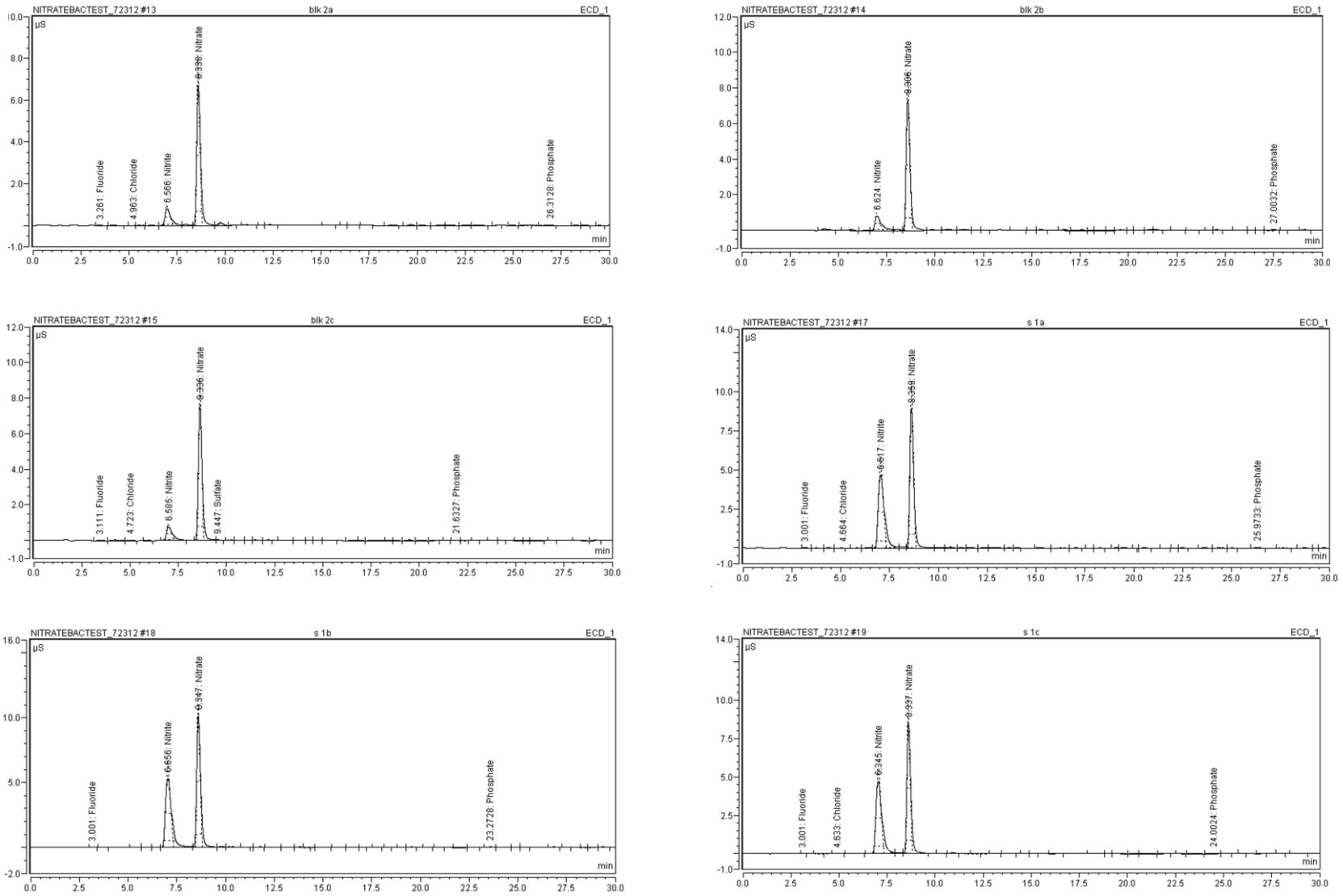


Figure A.24. Chromatograms of blk 2a, blk 2b, blk 2c, s 1a, s 1b, and s 1c after 168 hours.

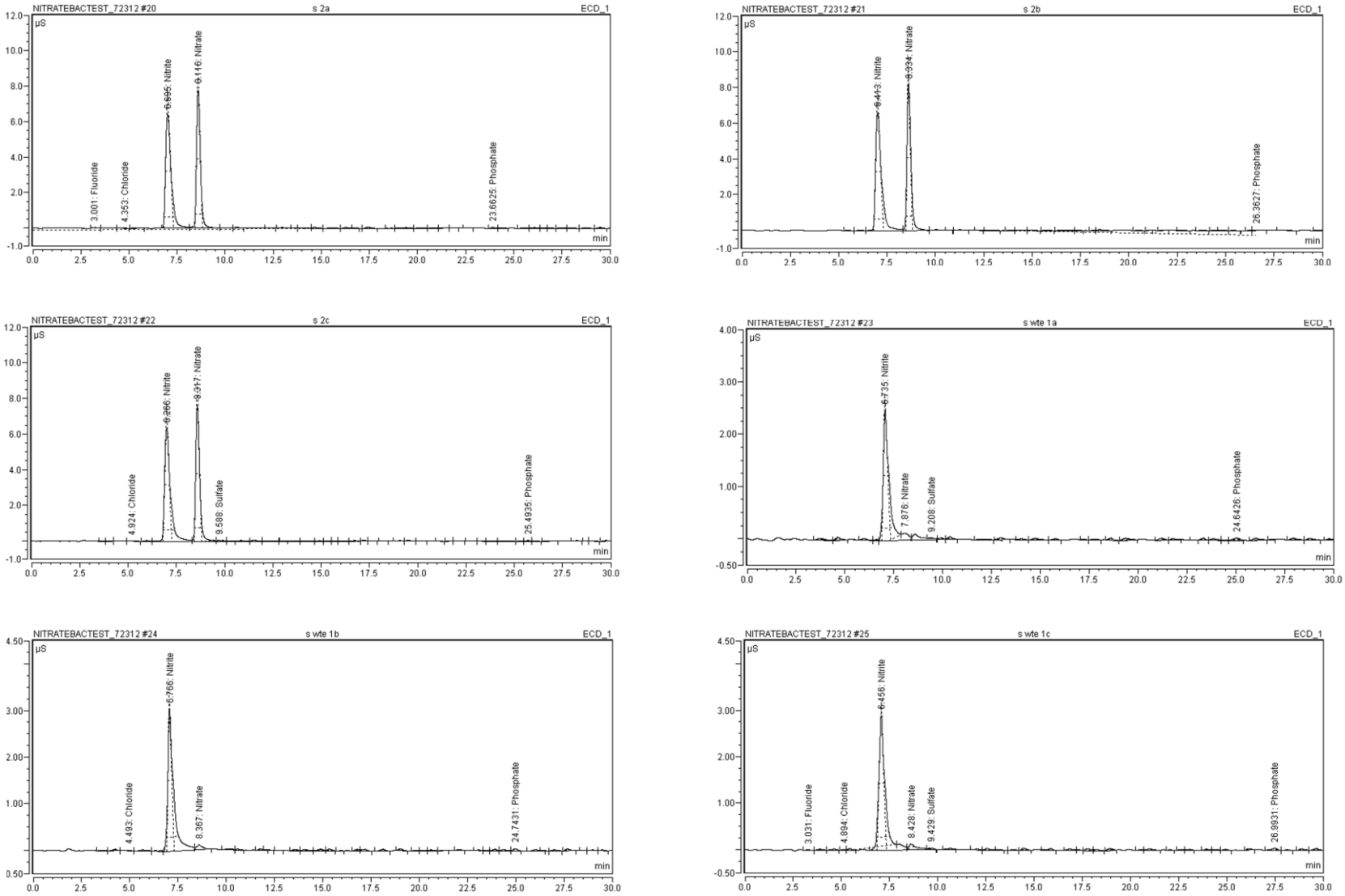


Figure A.25. Chromatograms of s 2a, s 2b, s 2c, s w TE 1a, s w TE 1b, and s w TE 1c after 168 hours.

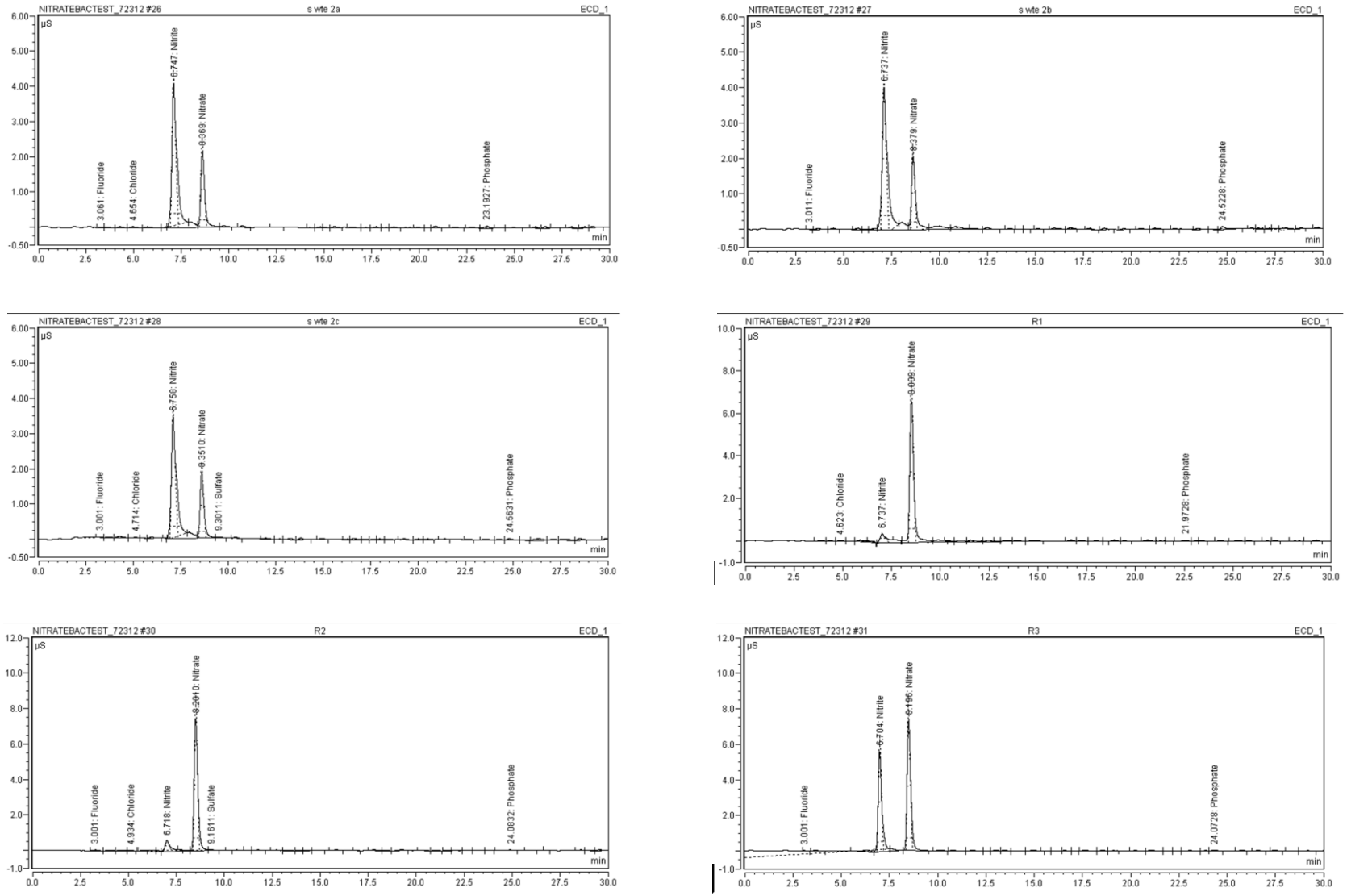


Figure A.26. Chromatograms of s w TE 2a, s w TE 2b, s w TE 2c, R 1, R 2, and R 3 after 168 hours.

APPENDIX B: COMPARISON OF NO_3-N UTILIZATION IN FREE CELLS VERSUS IMMOBILIZED CELLS DATA TABLES AND CHROMATOGRAMS

Table B.1. Sample code table for comparison of NO_3-N utilization in free cells versus immobilized cells samples.

Control Samples	**Code**
blank 1	blk 1
blank 2*	blk 2
Treatment Samples	
free cells, replicate 1	fc 1
free cells, replicate 2	fc 2
free cells, replicate 3	fc 3
inoculated beads, replicate 1	bac bd 1 or mf bd 1
inoculated beads, replicate 2	bac bd 2 or mf bd 2
Reservoir Samples	
NO_3-N stock solution, initial concentration, replicates 1-3	NO3 initial
NO_3-N stock solution (diluted 10x), replicate 1, refrigerated	stock 1 or stk 1
NO_3-N stock solution (diluted 10x), replicate 2, refrigerated	stock 2 or stk 2
NO_3-N stock solution (undiluted), replicate 1, refrigerated	stk undil 1
NO_3-N stock solution (undiluted), replicate 2, refrigerated	stk undil 2
Standard	
Dionex® Seven Anion Standard	7A
*blank 2 = contaminated with *Methylobacterium fujisawaense*	

Table B.2. Free cells versus immobilized cells NO_3-N concentrations and corresponding chromatogram locations.

Sample Code	Hour	NO_3-N(mg/L)	Chromatogram Appendix #	Appendix page #
7A	0	22.6	Figure B.1 (starting with 7A in the top left, moving left to right down the page, ending with blk 1 in the bottom right)	94
NO3 initial	0	10.0		
NO3 initial	0	10.0		
NO3 initial	0	10.1		
7A	24	22.6		
blk 1	24	10.0		
blk 2	24	10.2	Figure B.2 (starting with blk 2 in the top left, moving left to right down the page, ending with stock 1 in the bottom right)	95
fc 1	24	9.5		
fc 2	24	10.3		
bac bd 1	24	7.0		
bac bd 2	24	6.5		
stock 1	24	10.3		
stock 2	24	10.5	Figure B.3 (starting with stock 2 in the top left, moving left to right down the page, ending with fc 2 in the bottom right)	96
7A	48	22.6		
blk 1	48	8.4		
blk 2	48	8.9		
fc 1	48	10.4		
fc 2	48	4.0		
fc 3	48	4.1	Figure B.4 (starting with fc 3 in the top left, moving left to right down the page, ending with 7A in the bottom right)	97
bac bd 1	48	3.9		
bac bd 2	48	3.1		
stock 1	48	8.0		
stock 2	48	8.0		
7A	72	22.6		
blk 1	72	8.0	Figure B.5 (starting with blk 1 in the top left, moving left to right down the page, ending with bac bd 1 in the bottom right)	98
blk 2	72	8.8		
fc 1	72	10.5		
fc 2	72	0.0		
fc 3	72	0.2		
bac bd 1	72	1.2		
bac bd 2	72	0.4	Figure B.6 (starting with bac bd 2 in the top left, moving left to right down the page,	99
stock 1	72	9.0		
stock 2	72	9.6		
7A	96	22.6		

(continued)

Table B.2. Free cells versus immobilized cells NO_3-N concentrations and corresponding chromatogram locations (continued).

Sample Code	Hour	NO_3-N(mg/L)	Chromatogram Appendix #	Appendix page #
blk 1	96	8.3	ending with blk 2 in the bottom right)	99
blk 2	96	9.4		
fc 1	96	10.8	Figure B.7 (starting with fc 1 in the top left, moving left to right down the page, ending with stk undil 1 in the bottom right)	100
fc 2	96	0.0		
fc 3	96	0.0		
mf bd 1	96	0.8		
mf bd 2	96	0.3		
stk undil 1	96	11.0		
stk undil 2	96	11.0	Figure B.8 (starting with stk undil 2 in the top left, moving left to right down the page, ending with blk 2 in the bottom right)	101
stk 1	96	7.6		
stk 2	96	8.0		
7A	120	22.6		
blk 1	120	7.5		
blk 2	120	7.5		
fc 1	120	9.5	Figure B.9 (starting with fc 1 in the top left, moving left to right down the page, ending with stk undil 1 in the bottom right)	102
fc 2	120	0.0		
fc 3	120	0.0		
mf bd 1	120	0.7		
mf bd 2	120	0.3		
stk undil 1	120	10.9		
stk undil 2	120	10.9	Figure B.10 (starting with stk undil 2 in the top left, moving left to right down the page, ending with blk 2 in the bottom right)	103
stk 1	120	9.0		
stk 2	120	9.0		
7A	144	22.6		
blk 1	144	11.9		
blk 2	144	7.6		
fc 1	144	12.7	Figure B.11 (starting with fc 1 in the top left, moving left to right down the page, ending with stk undil 1 in the bottom right)	104
fc 2	144	11.4		
fc 3	144	0.3		
mf bd 1	144	4.6		
mf bd 2	144	4.4		
stk undil 1	144	11.1		
stk undil 2	144	11.0	Figure B.12 (starting with stk undil 2 in the top left,	105
stk 1	144	3.2		
stk 2	144	3.6		

(continued)

Table B.2. Free cells versus immobilized cells NO_3-N concentrations and corresponding chromatogram locations (continued).

Sample Code	Hour	NO_3-N(mg/L)	Chromatogram Appendix #	Appendix page #
7A	168	22.6	moving left to right down the page, ending with blk 2 in the bottom right)	105
blk 1	168	9.2		
blk 2	168	2.2		
fc 1	168	10.1	Figure B.13 (starting with fc 1 in the top left, moving left to right down the page, ending with stk undil 1 in the bottom right)	106
fc 2	168	0.0		
fc 3	168	0.0		
mf bd 1	168	0.6		
mf bd 2	168	0.5		
stk undil 1	168	10.6		
stk undil 2	168	10.6	Figure B.14 (starting with stk undil 2 in the top left, moving left to right down the page, ending with stk 2 in the bottom right)	107
stk 1	168	8.6		
stk 2	168	8.1		

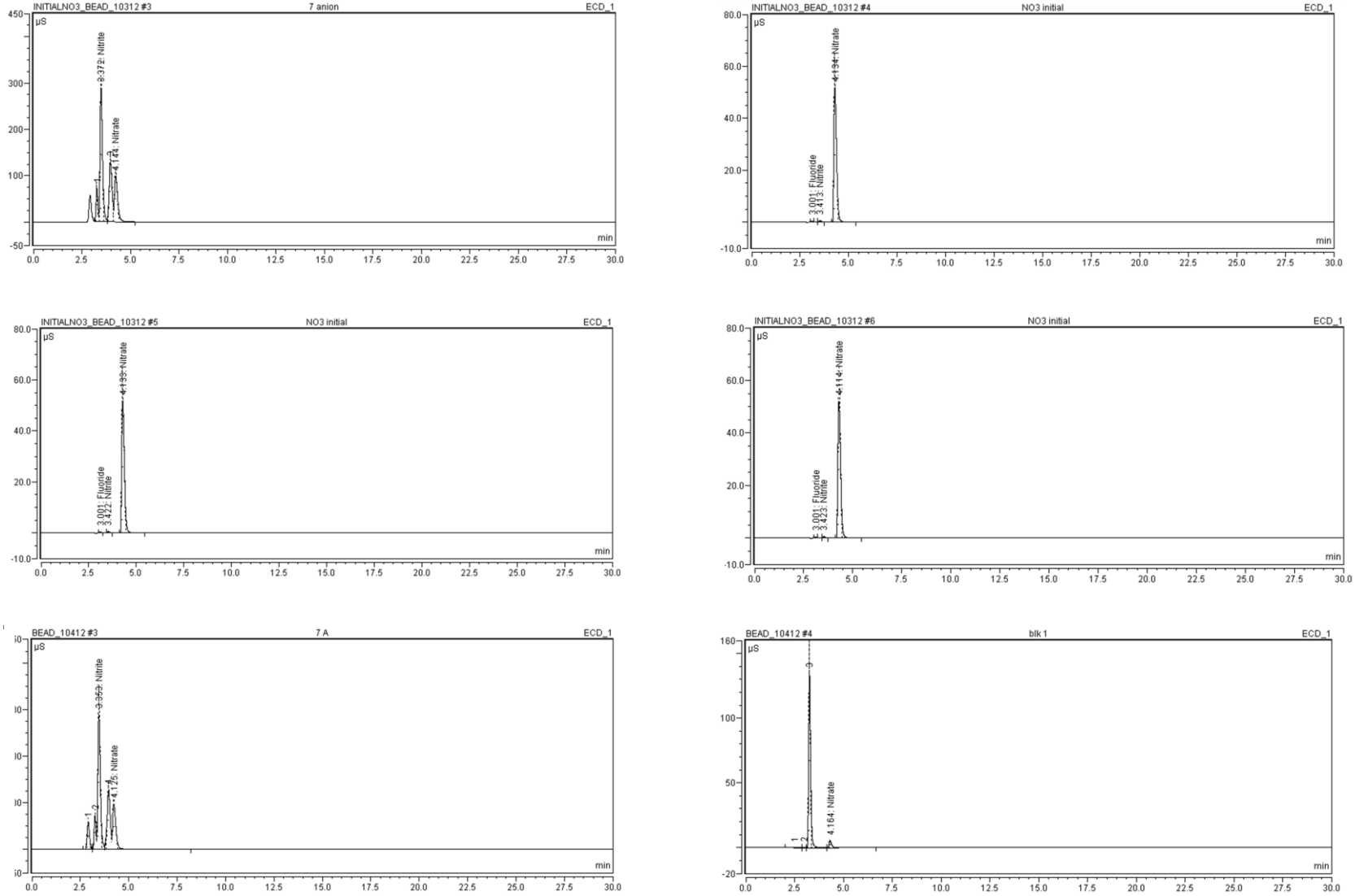


Figure B.1. Chromatograms of 7A (after 0 hours), NO3 initial (3 duplicates after 0 hours), 7A, and blk 1 after 24 hours.

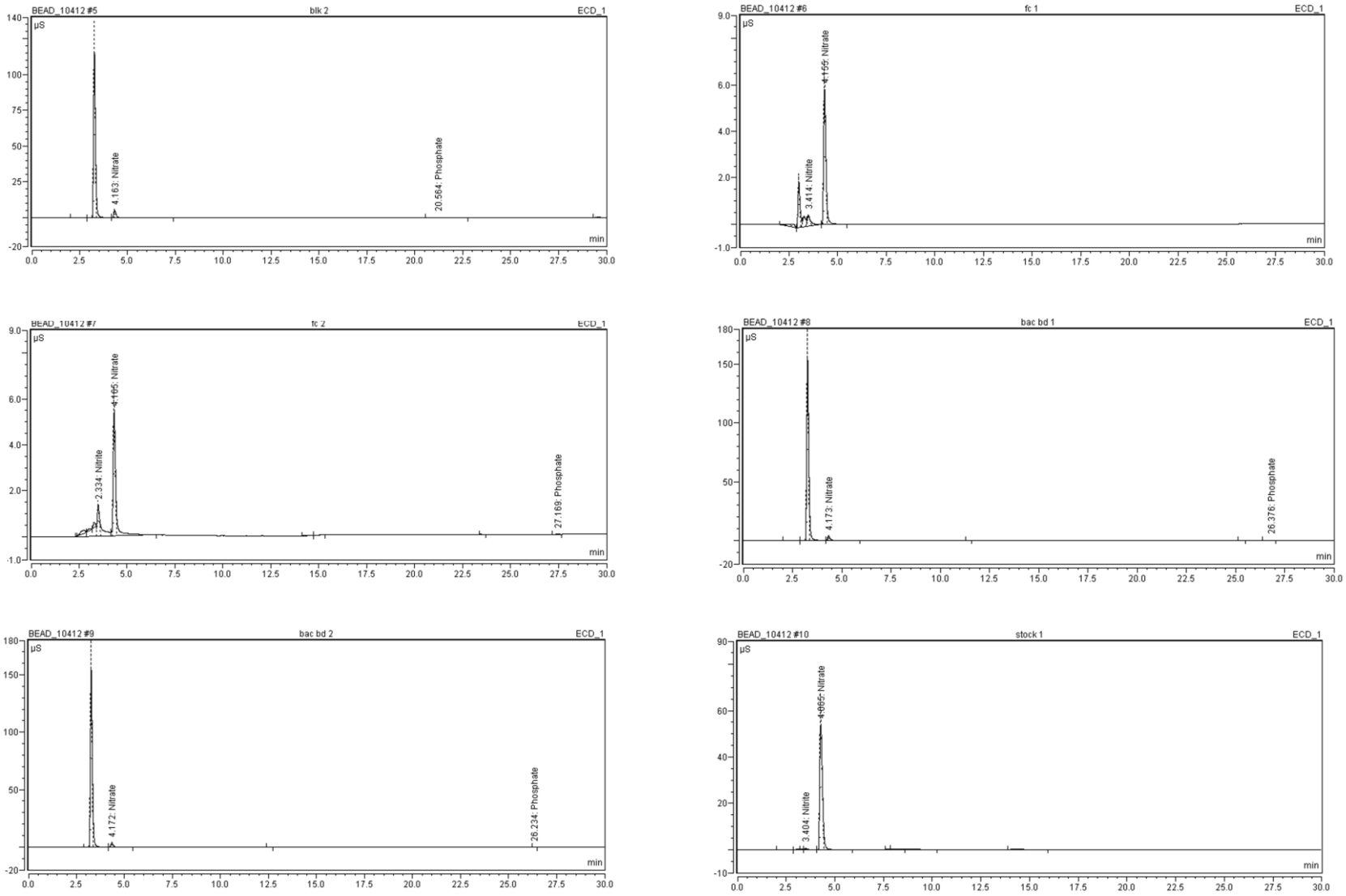


Figure B.2. Chromatograms of blk 2, fc 1, fc 2, bac bd 1, bac bd 2, and stock 1 after 24 hours.

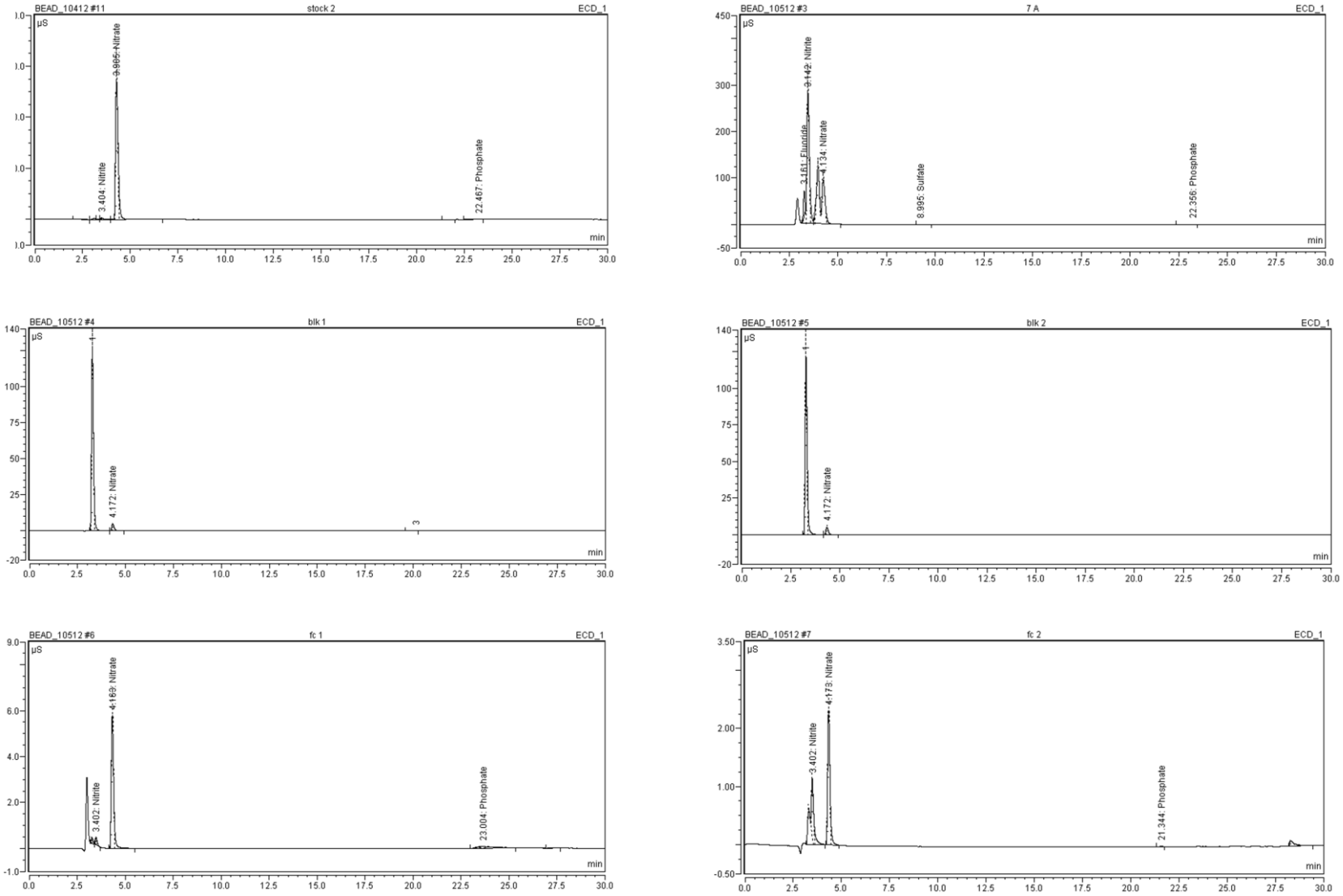


Figure B.3. Chromatograms of stock 2 (after 24 hours), 7A, blk 1, blk 2, fc 1, and fc 2 after 48 hours.

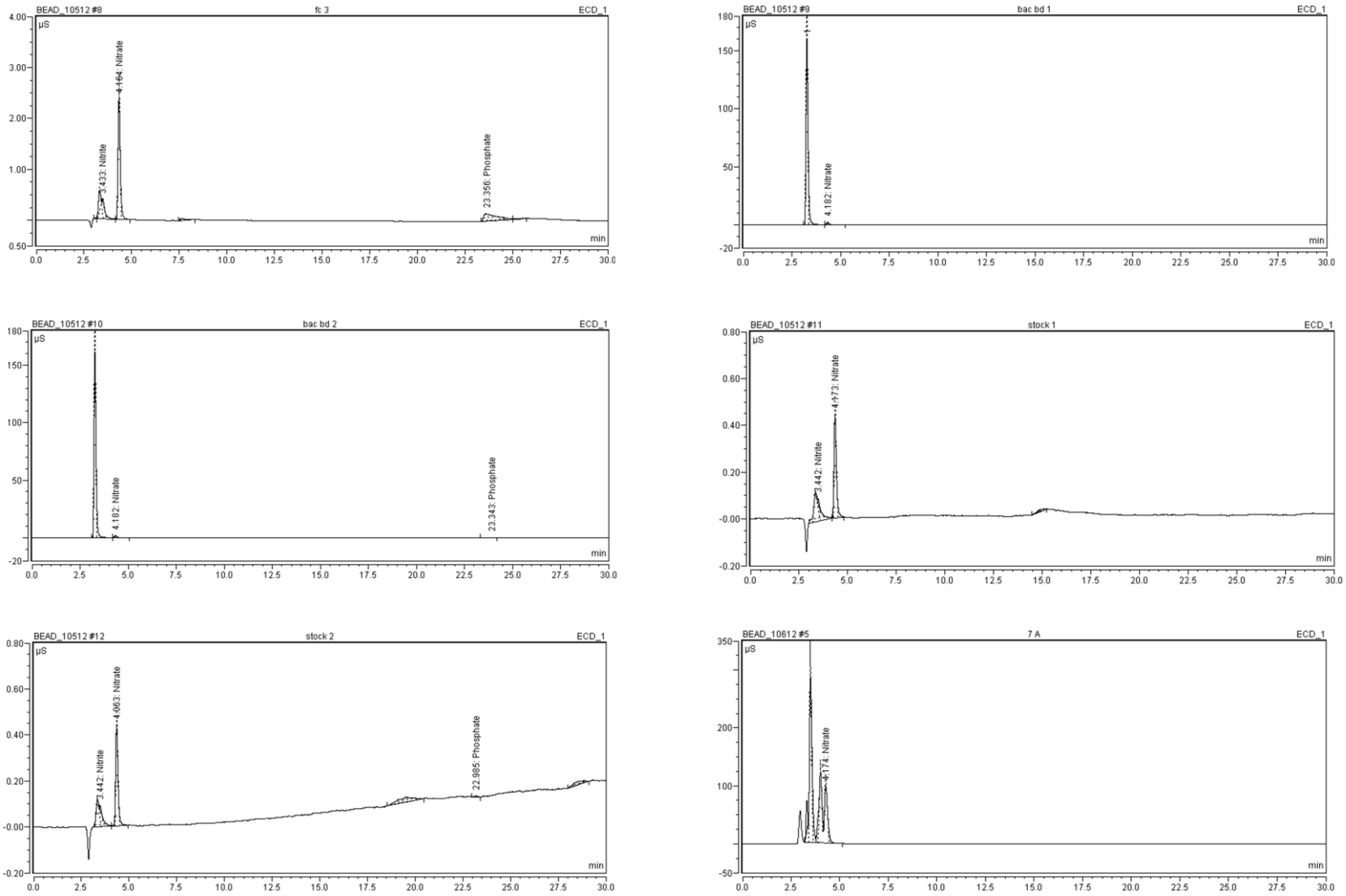


Figure B.4. Chromatograms of fc 3, bac bd 1, bac bd 2, stock 1, stock 2 (after 48 hours), and 7A (after 72 hours).

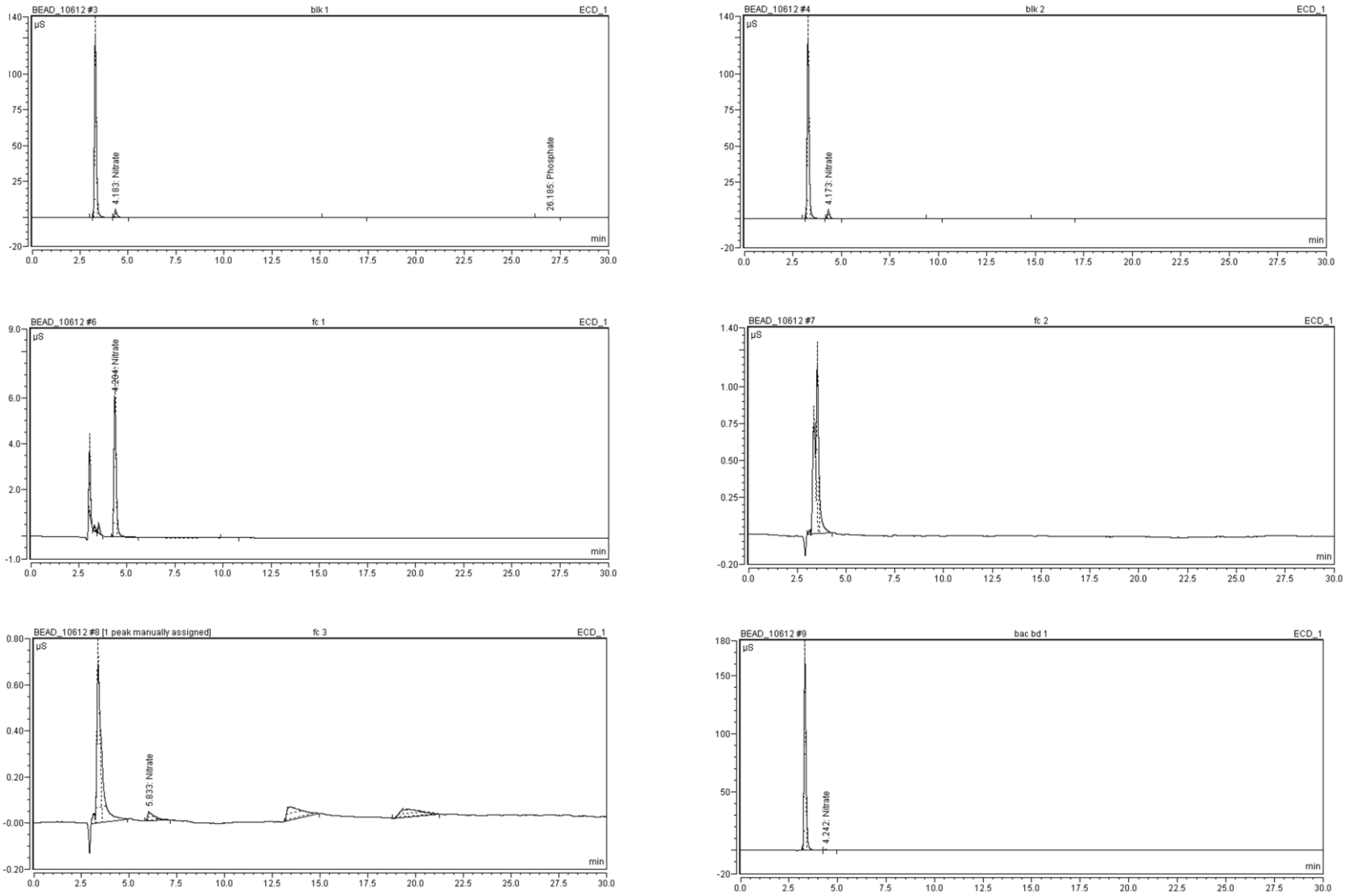


Figure B.5. Chromatograms of blk 1, blk 2, fc 1, fc 2, fc 3, and bac bd 1 after 72 hours.

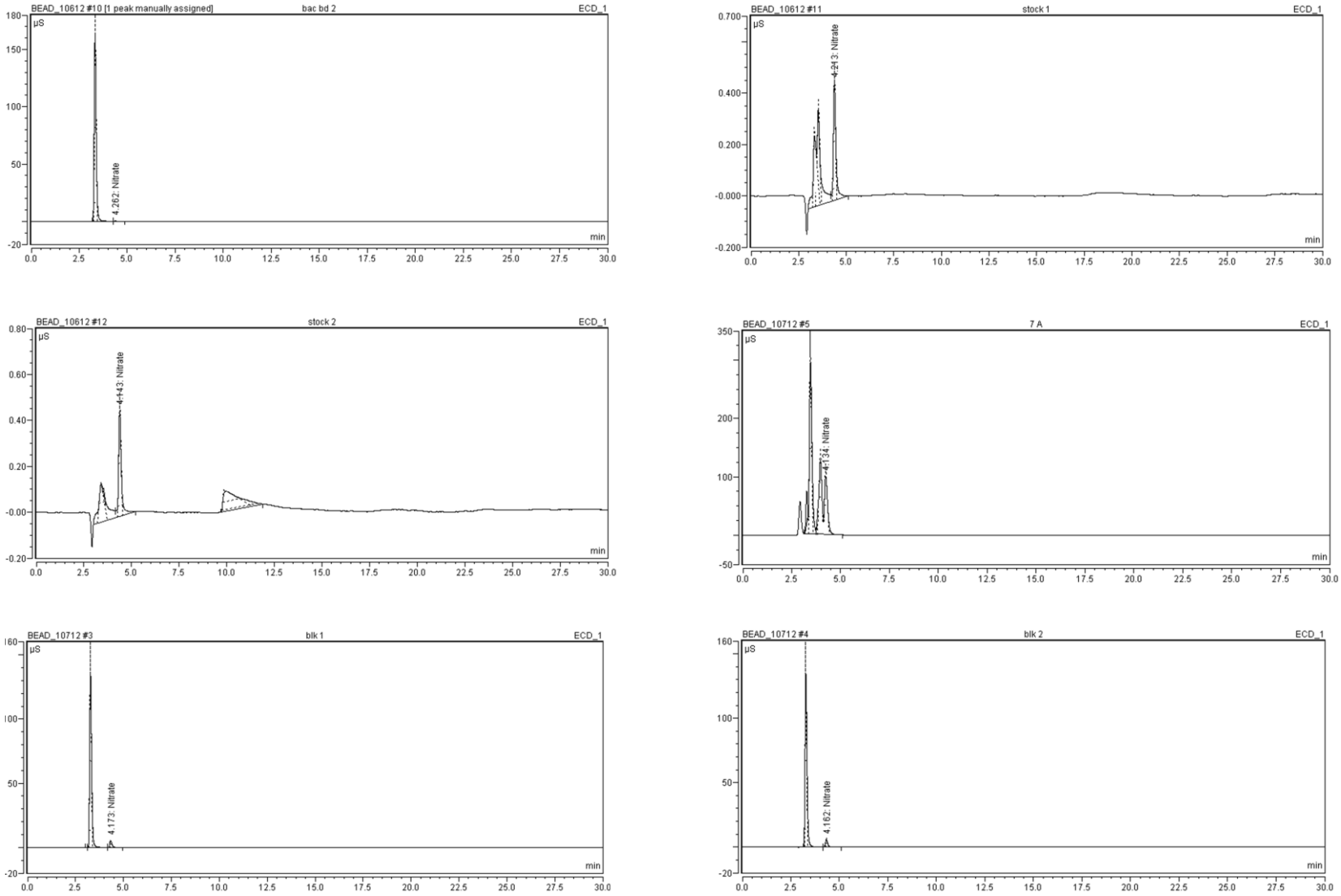


Figure B.6. Chromatograms of bac bd 2 (after 72 hours), stock 1 (after 72 hours), stock 2 (after 72 hours), 7A (after 96 hours), blk 1 (after 96 hours), and blk 2 (after 96 hours).

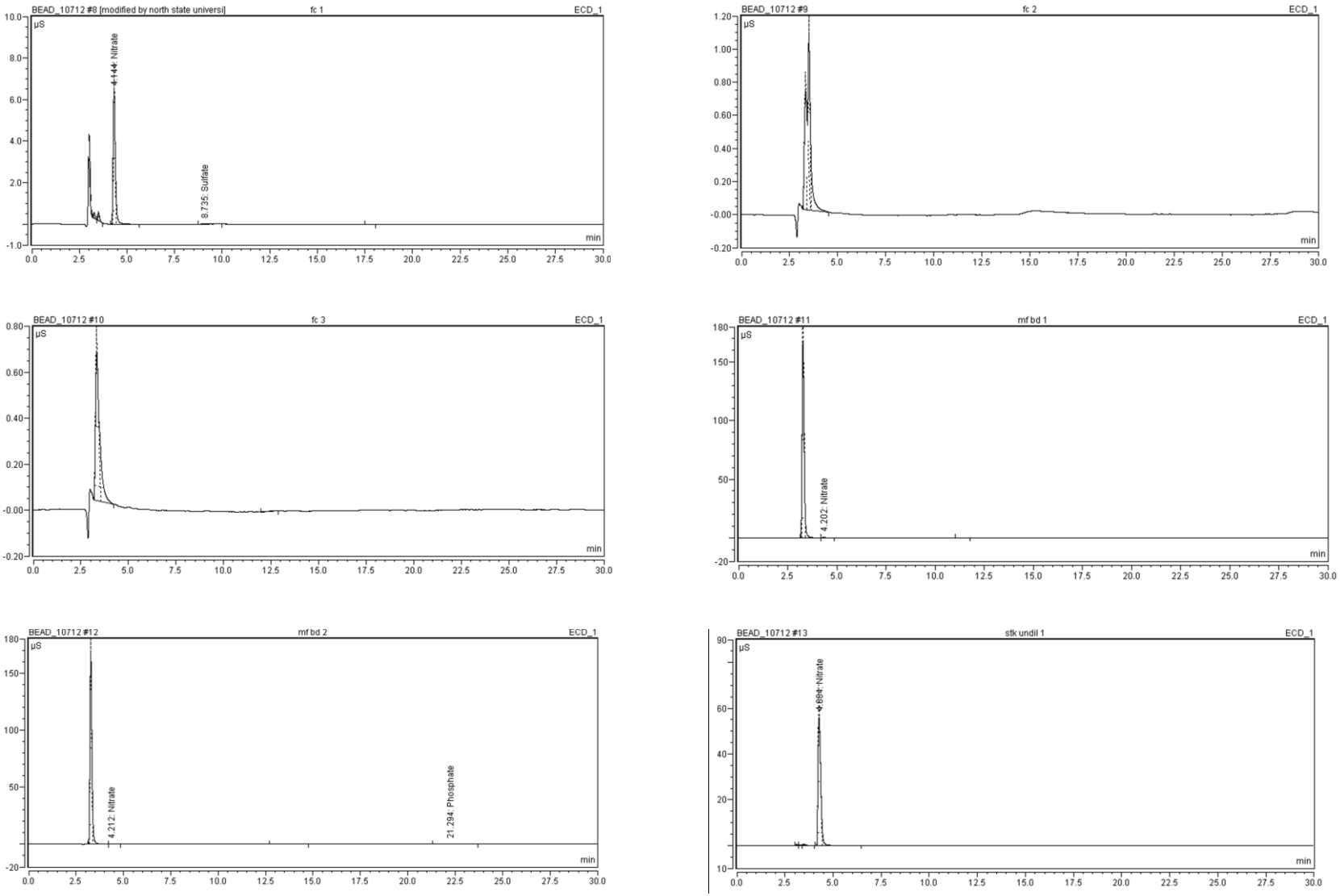


Figure B.7. Chromatograms of fc 1, fc 2, fc 3, mf bd 1, mf bd 2, and stk undil 1 after 96 hours.

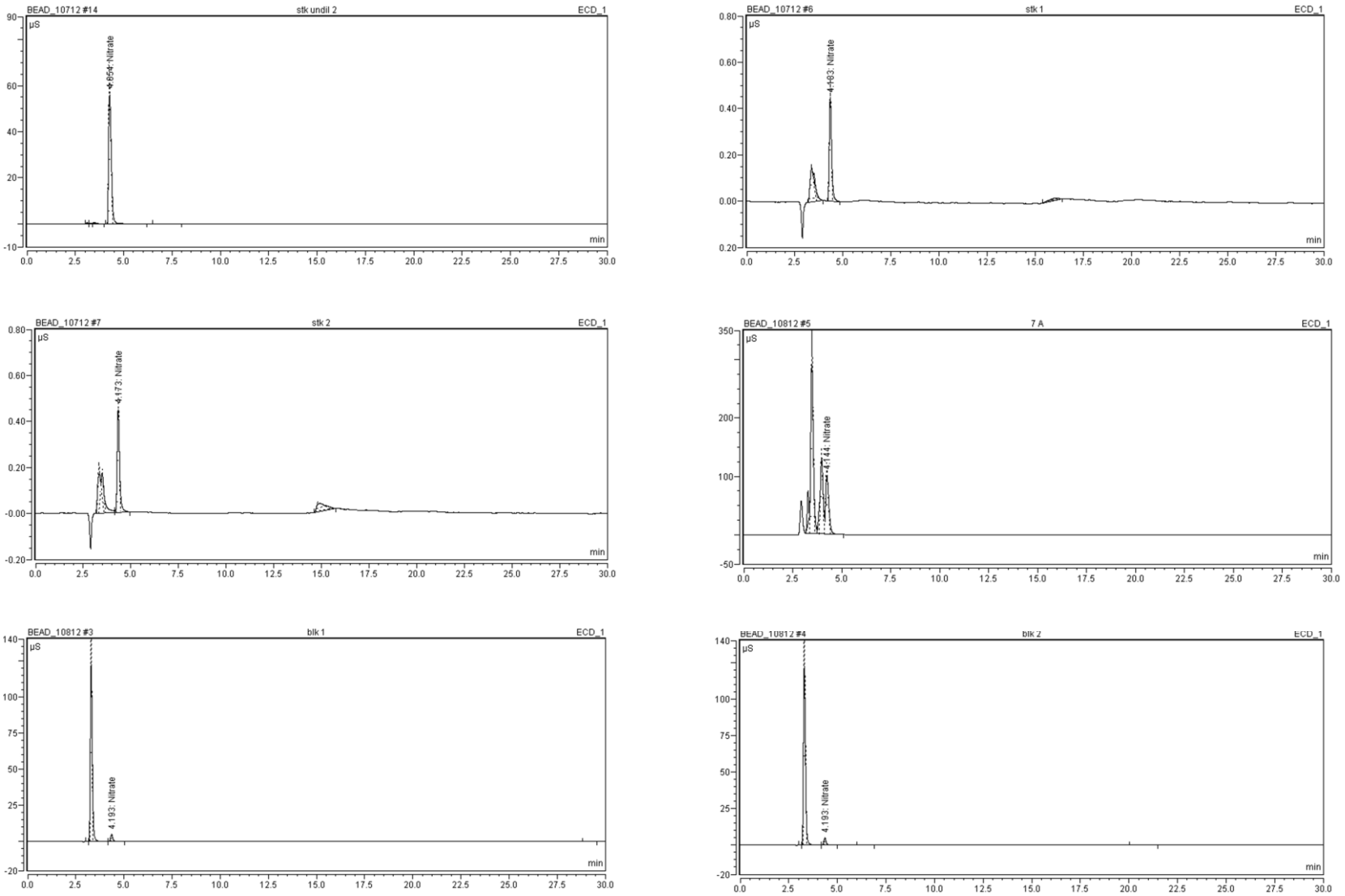


Figure B.8. Chromatograms of stk undil 2 (after 96 hours), stk 1 (after 96 hours), stk 2 (after 96 hours), 7A (after 120 hours), blk 1 (after 120 hours), and blk 2 (after 120 hours).

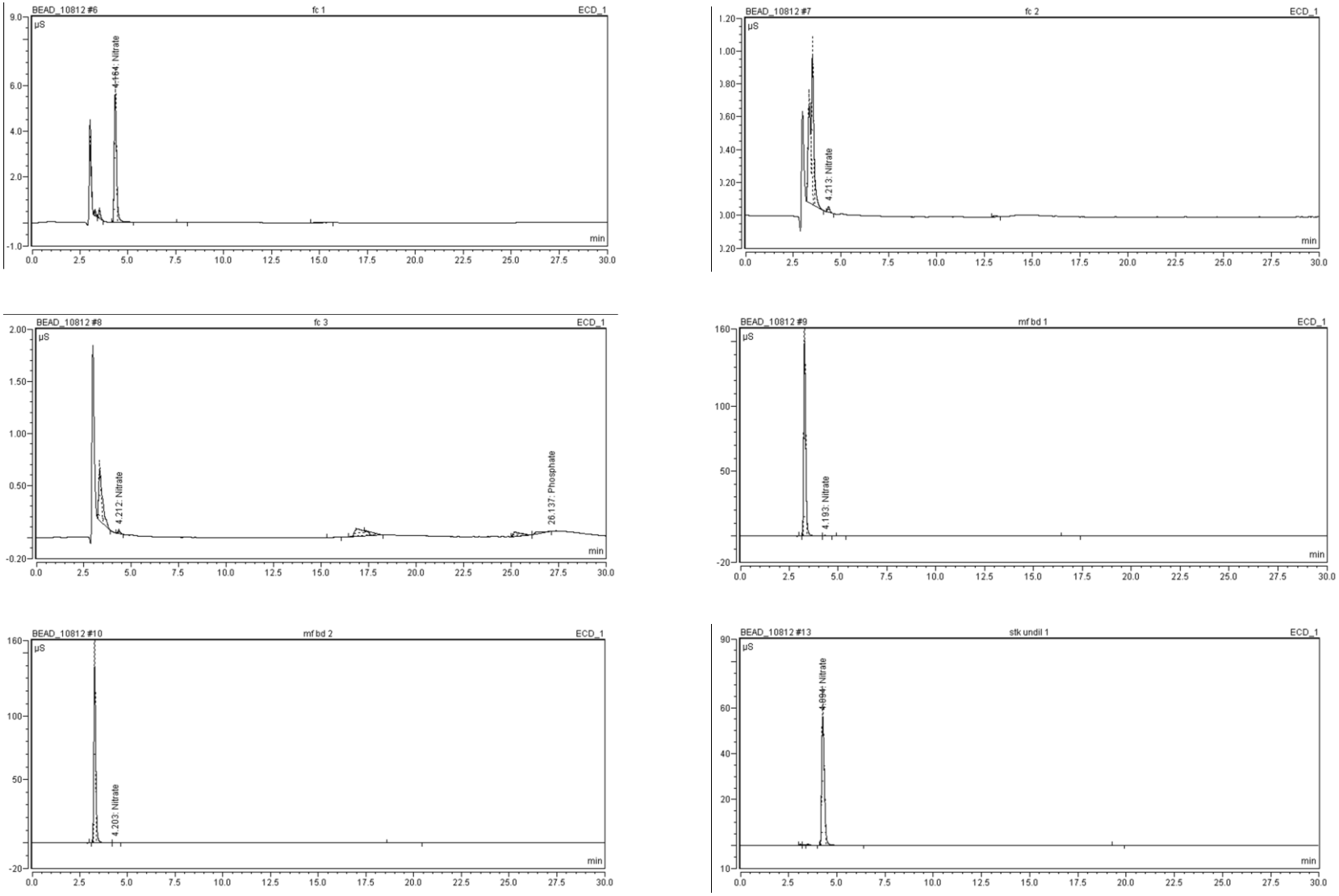


Figure B.9. Chromatograms of fc 1, fc 2, fc 3, mf bd 1, mf bd 2, and stk undil 1 after 120 hours.

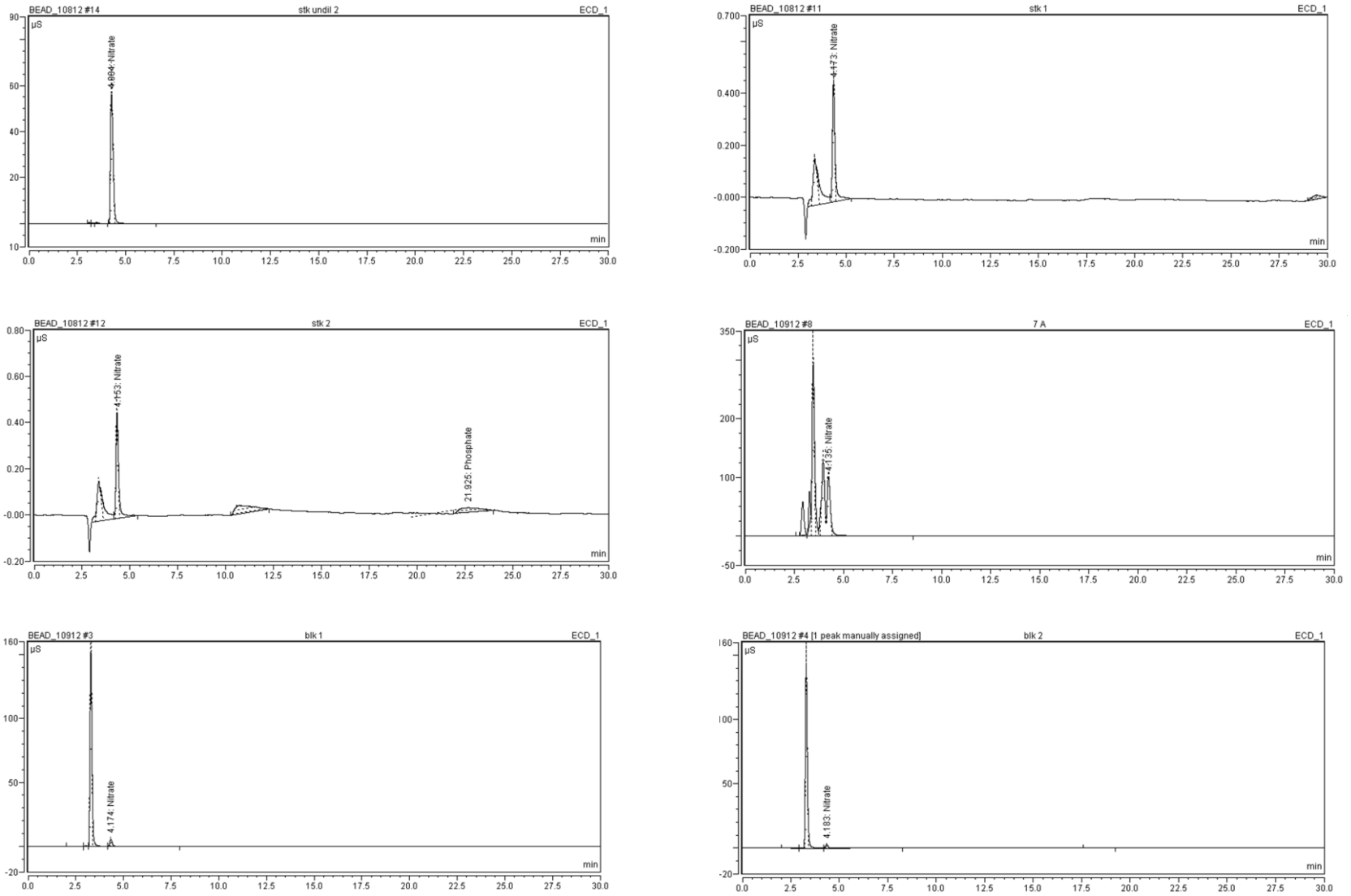


Figure B.10. Chromatograms of stk undil 2 (after 120 hours), stk 1 (after 120 hours), stk 2 (after 120 hours), 7A (after 144 hours), blk 1 (after 144 hours), and blk 2 (after 144 hours).

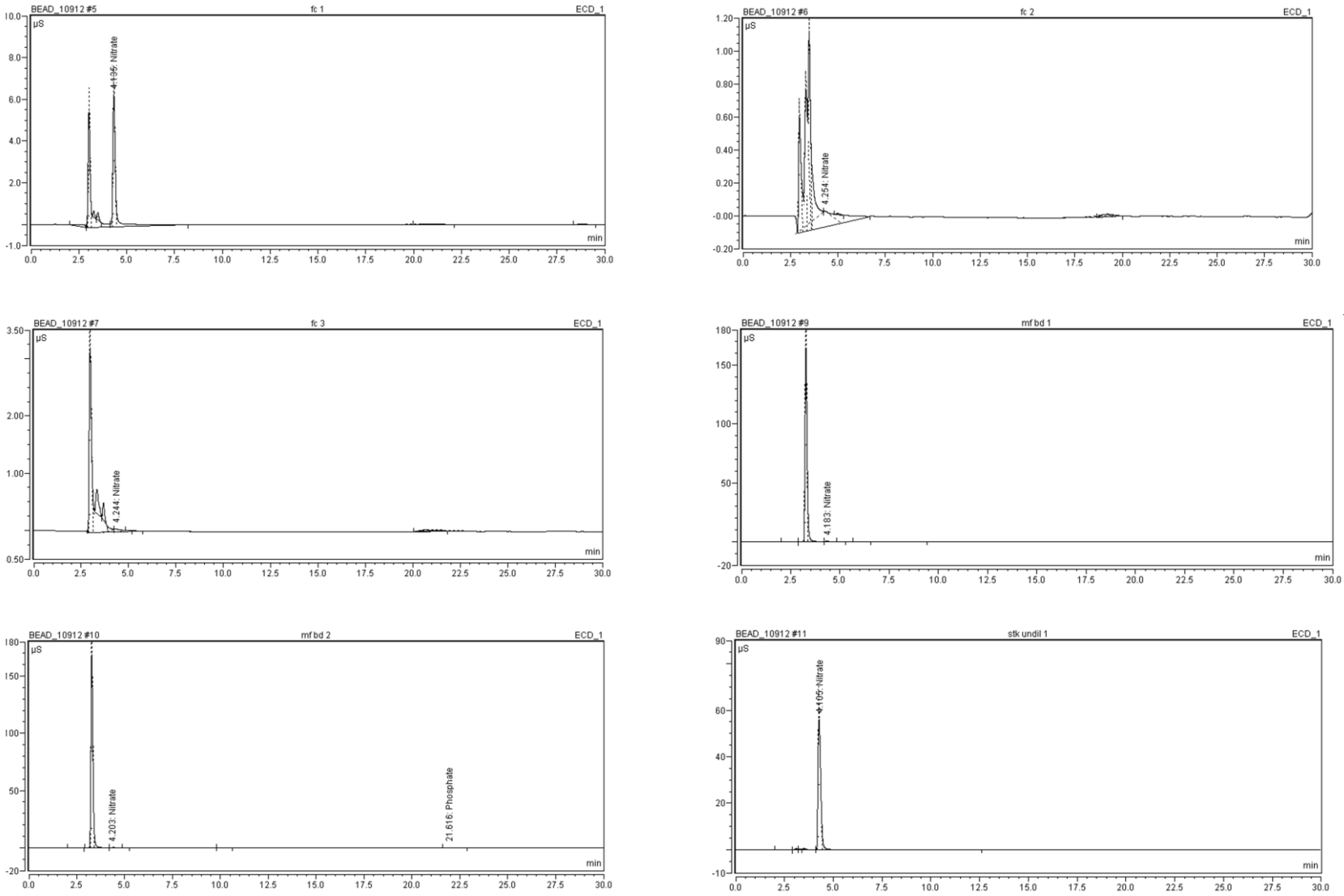


Figure B.11. Chromatograms of fc 1, fc 2, fc 3, mf bd 1, mf bd 2, and stk undil 1 after 144 hours.

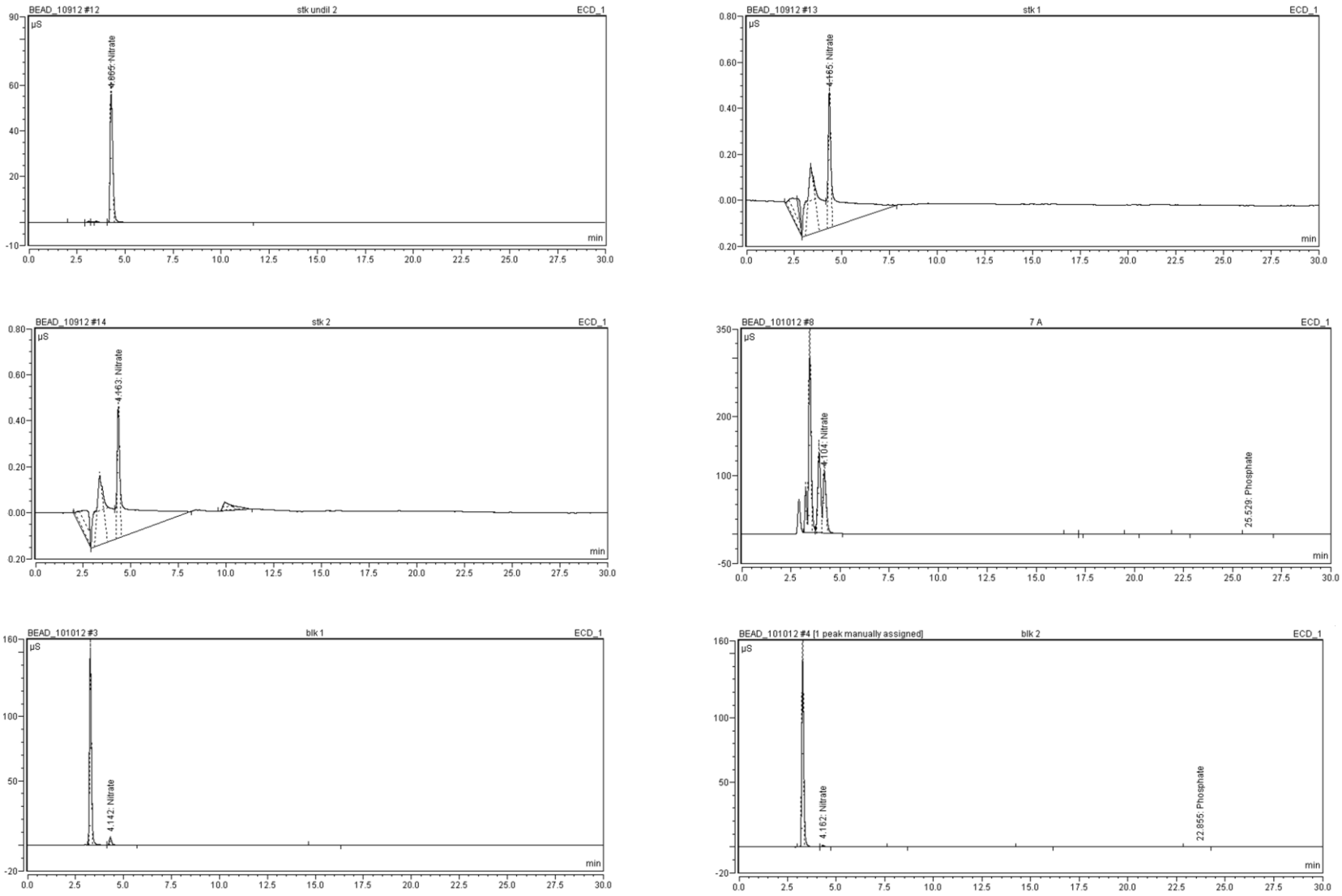


Figure B.12. Chromatograms of stk undil 2 (after 144 hours), stk 1 (after 144 hours), stk 2 (after 144 hours), 7A (after 168 hours), blk 1 (after 168 hours), and blk 2 (after 168 hours).

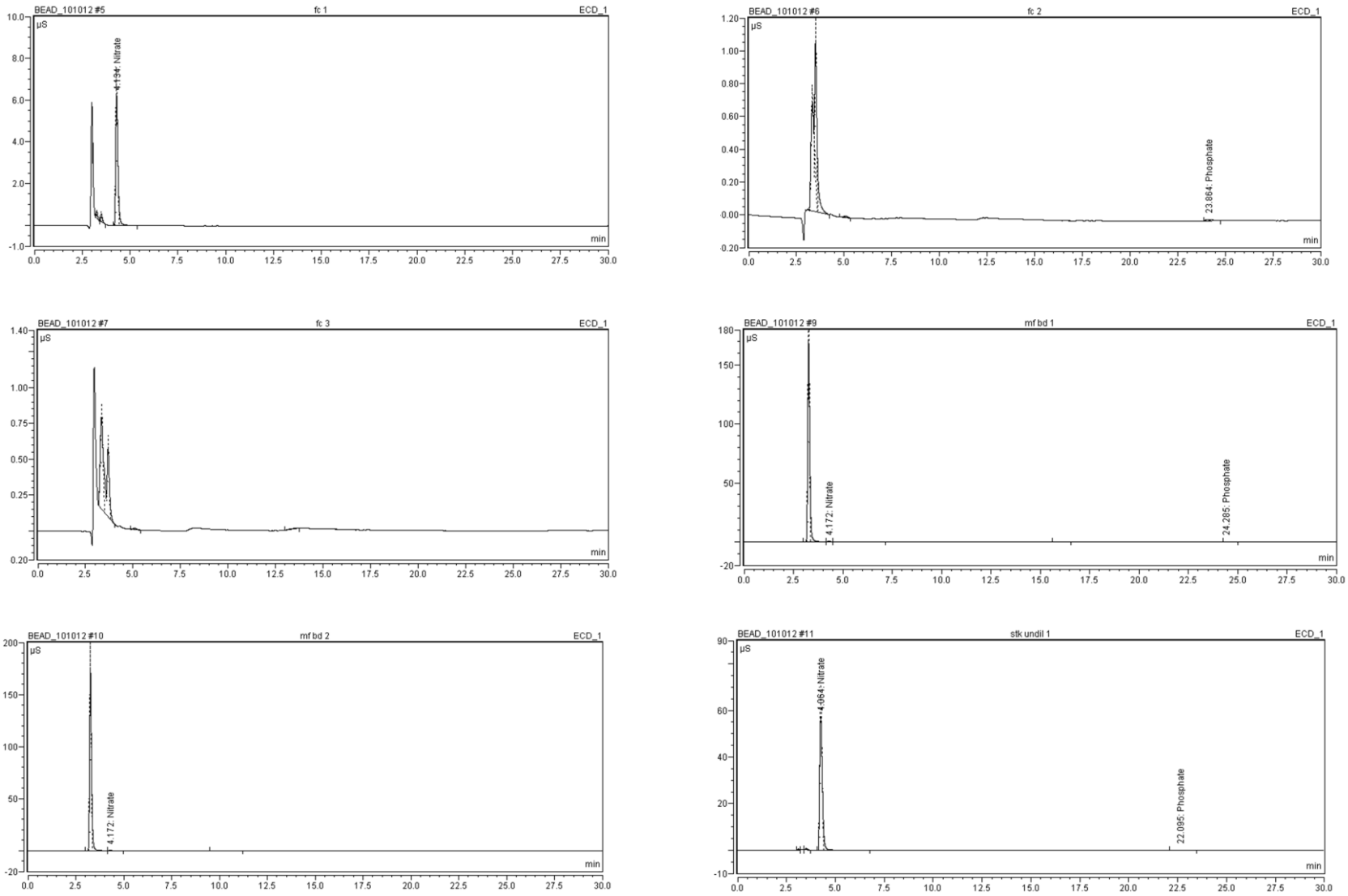


Figure B.13. Chromatograms of fc 1, fc 2, fc 3, mf bd 1, mf bd 2, and stk undil 1 after 168 hours.

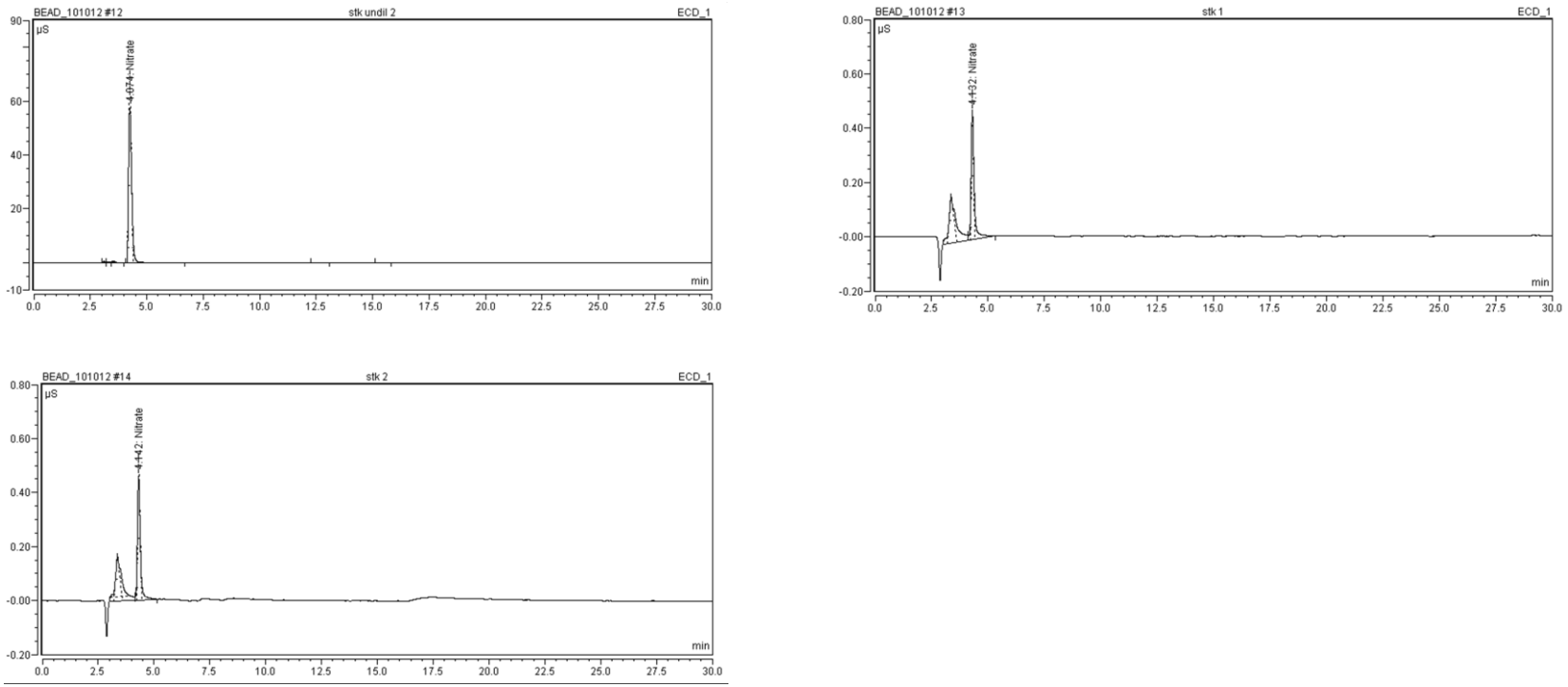


Figure B.14. Chromatograms of stk undil 2, stk 1, and stk 2 after 168 hours.

APPENDIX C: COMPARISON OF NO_3-N UTILIZATION IN FREE CELLS VERSUS IMMOBILIZED CELLS WITH AERATION DATA TABLES AND CHROMATOGRAMS

Table C.1. Sample code table for comparison of NO_3-N utilization in free cells versus immobilized cells samples.

Control Sample	**Code**
blank beads	blank
Treatment Samples	
inoculated beads	bds
free cells	free
Reservoir Samples	
NO_3-N stock solution, initial concentration, blank beads	initial blks
NO_3-N stock solution, initial concentration, inoculated beads	initial bds
NO_3-N stock solution, initial concentration, free cells	initial free
Standard	
Dionex® Seven Anion Standard	7A

Table C.2. Comparison of NO_3-N utilization in free cells versus immobilized cells with aeration NO_3-N concentrations and corresponding chromatogram locations.

Chromatogram Label	Sample Code	Trial #	Hour	NO_3-N(mg/L)	Chromatogram Appendix #	Appendix page #
7a	7A	1	0	22.3	Figure C.1 (starting with 7A in the top left, moving left to right down the page, ending with 1221 bds in the bottom right)	112
initial bds	initial bds	1	0	8.4		
initial free	initial free	1	0	9.8		
1220 bds	bds	1	24	7.7		
1220 free	free	1	24	9.0		
1221 bds	bds	1	48	4.5		
1221 free	free	1	48	6.1	Figure C.2 (starting with 1221 free in the top left, moving left to right down the page, ending with 1224 bds in the bottom right)	113
1222 bds	bds	1	72	0.1		
1222 free	free	1	72	2.1		
1223 bds	bds	1	96	0.2		
1223 free	free	1	96	1.4		
1224 bds	bds	1	120	0.2		
1224 free	free	1	120	1.2	Figure C.3 (starting with 1224 free in the top left, moving left to right down the page, ending with 1227 bds in the bottom right)	114
1225 bds	bds	1	144	0.2		
1225 free	free	1	144	1.2		
1226 bds	bds	1	168	0.2		
1226 free	free	1	168	1.2		
1227 bds	bds	1	192	0.2		
1227 free	free	1	192	1.3	Figure C.4 (starting with 1227 free in the top left, moving left to right down the page, ending with blank 9 in the bottom right)	115
7 anion	7A	2	0	22.3		
initial blank 8	initial blks	2	0	9.4		
initial bds 8	initial bds	2	0	8.9		
initial free 8	initial free	2	0	9.7		
blank 9	blank	2	24	9.5		
bds 9	bds	2	24	7.2	Figure C.5 (starting with bds 9 in the top left, moving left to right down the page, ending with blank 11 in the bottom right)	116
free 9	free	2	24	9.0		
blank 10	blank	2	48	9.8		
bds 10	bds	2	48	4.0		
free 10	free	2	48	6.9		
blank 11	blank	2	72	10.0		
bds 11	bds	2	72	1.7	Figure C.6 (starting with bds 11	117
free 11	free	2	72	2.6		

(continued)

Table C.2. Comparison of NO_3-N utilization in free cells versus immobilized cells with aeration NO_3-N concentrations and corresponding chromatogram locations (continued).

Chromatogram Label	Sample Code	Trial #	Hour	NO_3-N(mg/L)	Chromatogram Appendix #	Appendix page #
blk 12	blank	2	96	10.3	in the top left, moving left to right down the page, ending with blk 13 in the bottom right)	117
bds 12	bds	2	96	1.7		
free 12	free	2	96	0.0		
blk 13	blank	2	120	10.5		
bds 13	bds	2	120	1.7	Figure C.7 (starting with bds 13 in the top left, moving left to right down the page, ending with blk 15 in the bottom right)	118
free 13	free	2	120	0.0		
blk 14	blank	2	144	10.9		
bds 14	bds	2	144	1.8		
free 14	free	2	144	0.0		
blk 15	blank	2	168	11.3		
bds 15	bds	2	168	1.9	Figure C.8 (starting with bds 15 in the top left, moving left to right down the page, ending with initial free in the bottom right)	119
free 15	free	2	168	0.0		
7 anion	7A	3	0	22.3		
initial blk	initial blks	3	0	11.5		
initial mf bds	initial bds	3	0	11.554		
initial free	initial free	3	0	9.7		
1 20 blk	blank	3	24	10.0	Figure C.9 (starting with 1 20 blk in the top left, moving left to right down the page, ending with 1 21 free in the bottom right)	120
1 20 mf bd	bds	3	24	7.9		
1 20 free	free	3	24	7.4		
1 21 blk	blank	3	48	10.2		
1 21 mf bd	bds	3	48	4.1		
1 21 free	free	3	48	3.5		
1 22 blk	blank	3	72	10.3	Figure C.10 (starting with 1 22 blk in the top left, moving left to right down the page, ending with 1 23 free in the bottom right)	121
1 22 mf bd	bds	3	72	0.4		
1 22 free	free	3	72	1.7		
1 22 blk	blank	3	96	10.4		
1 23 mf bd	bds	3	96	0.0		
1 23 free	free	3	96	0.9		
1 24 blk	blank	3	120	9.7	Figure C.11 (starting with 1 24 blk in the top left, moving left to right down the page,	122
1 24 mf bd	bds	3	120	0.0		
1 24 free	free	3	120	0.5		
1 25 blk	blank	3	144	8.8		
1 25 mf bd	bds	3	144	0.0		

(continued)

Table C.2. Comparison of NO_3-N utilization in free cells versus immobilized cells with aeration NO_3-N concentrations and corresponding chromatogram locations (continued).

Chromatogram Label	Sample Code	Trial #	Hour	NO_3-N(mg/L)	Chromatogram Appendix #	Appendix page #
free 1/25 6x dil	free	3	144	0.4	ending with free 1/25 6x dil in the bottom right)	122
blk 1/26	blank	3	168	8.3	Figure C.12 (starting with blk 1/26 in the top left, moving left to right down the page, ending with free 1/26 in the bottom right)	123
mf bds 1/26	bds	3	168	0.2		
free 1/26	free	3	168	0.1		

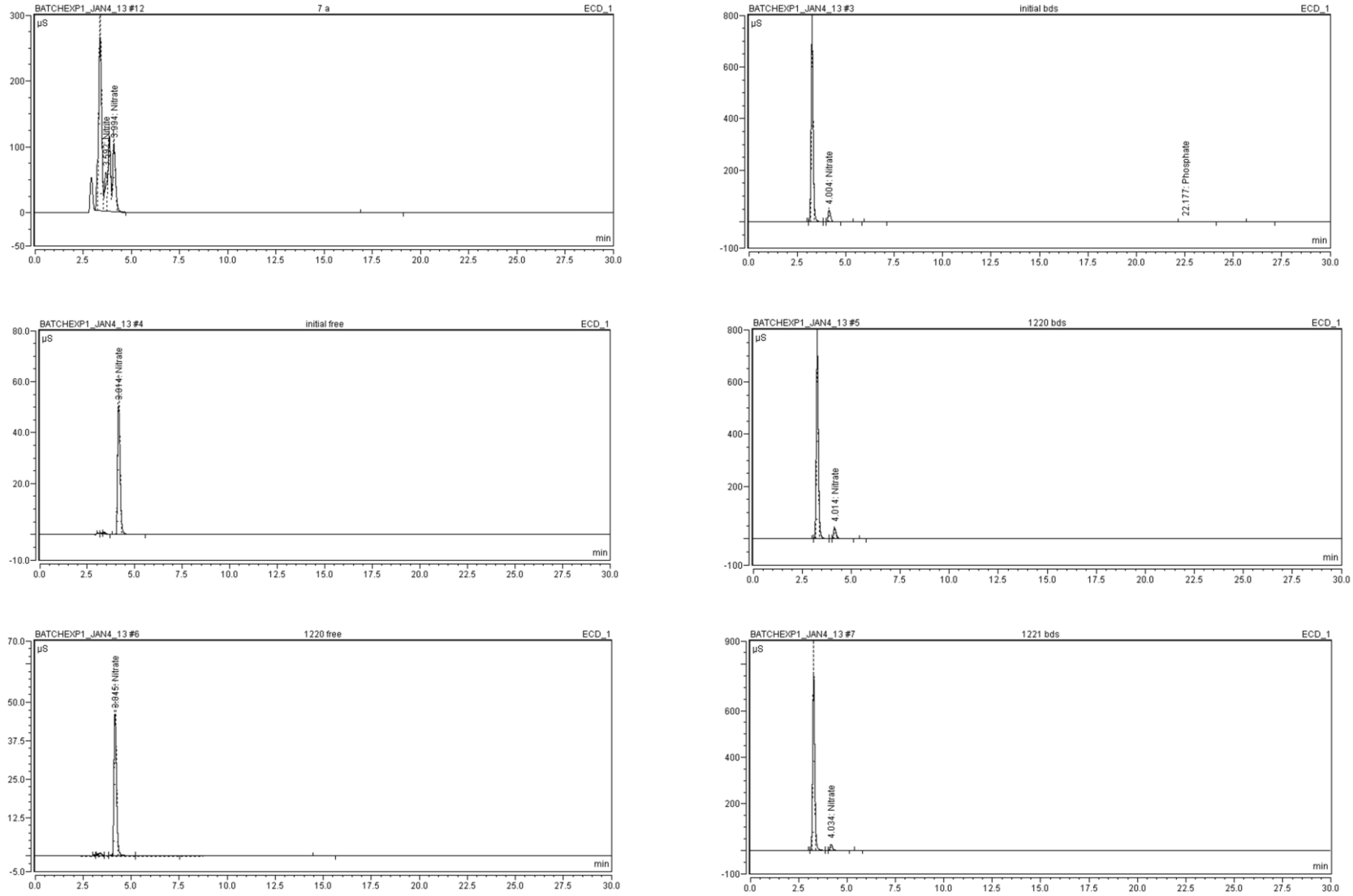


Figure C.1. Chromatograms of 7A (after 0 hours), initial bds (after 0 hours), initial free (after 0 hours), 1220 bds (after 24 hours), 1220 free (after 24 hours), and 1221 bds (after 48 hours).

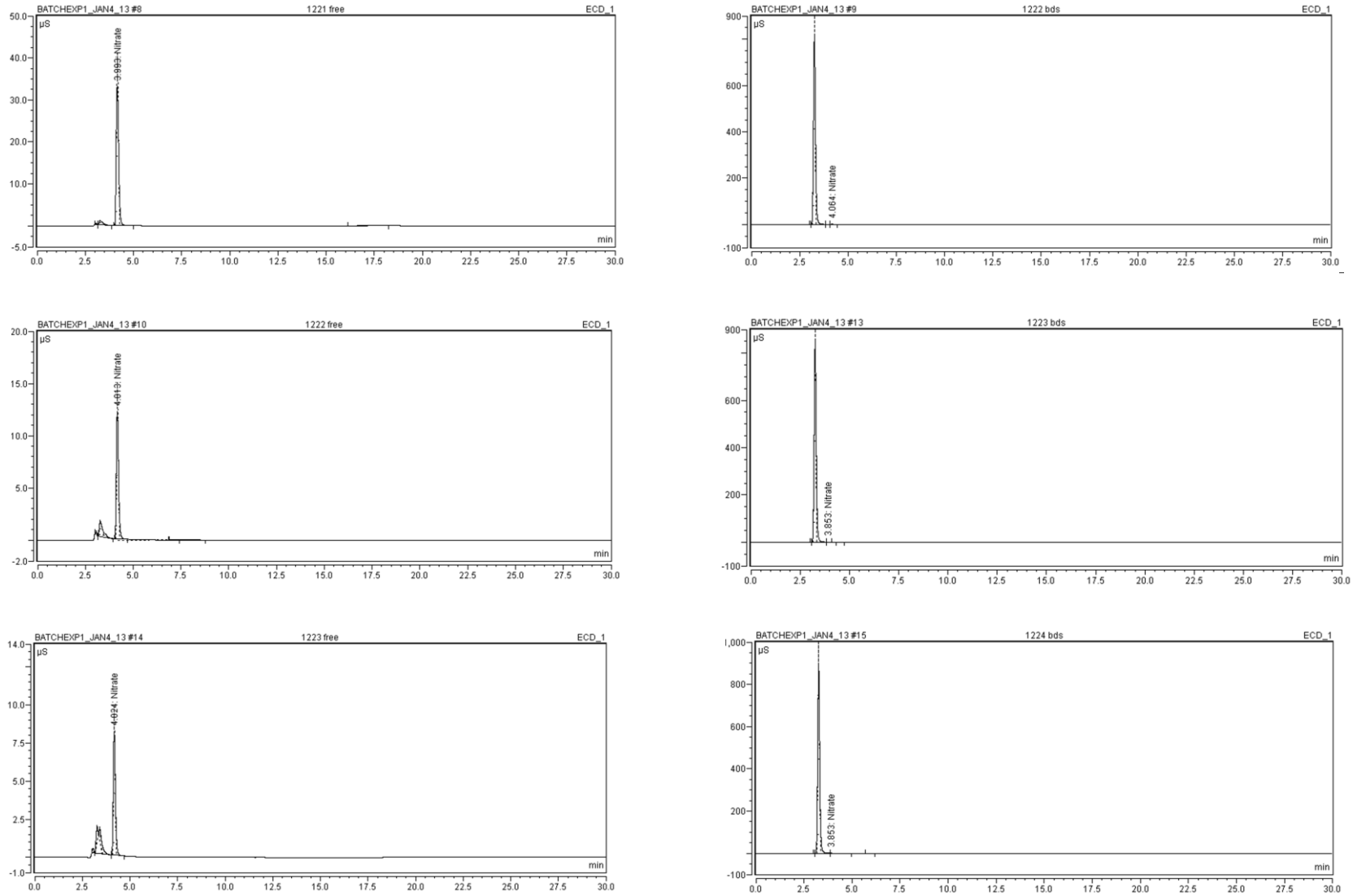


Figure C.2. Chromatograms of 1221 free (after 48 hours), 1222 bds (after 72 hours), 1222 free (after 72 hours), 1223 bds (after 96 hours), 1223 free (after 96 hours), and 1224 bds (after 120 hours).

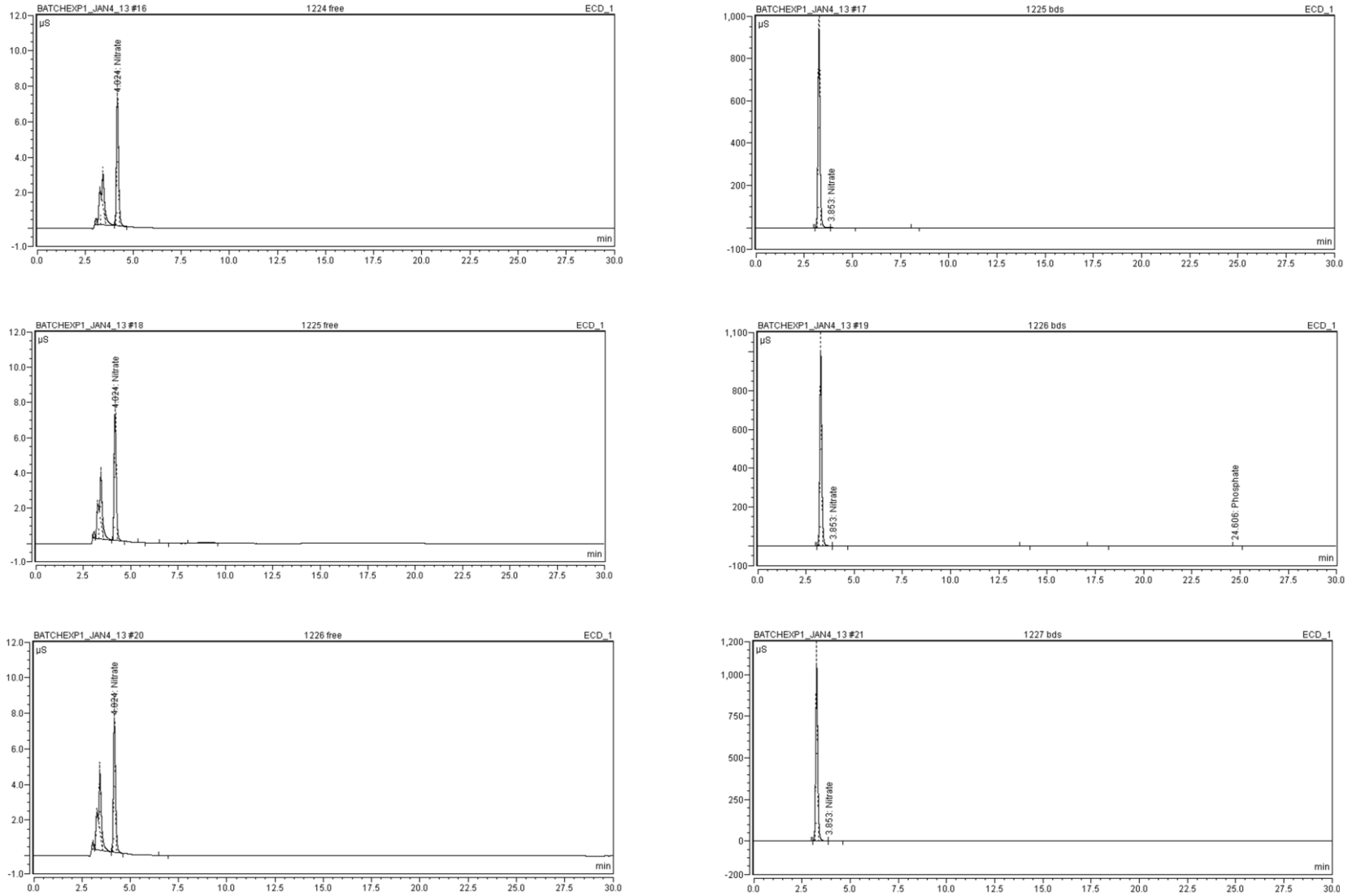


Figure C.3. Chromatograms of 1224 free (after 120 hours), 1225 bds (after 144 hours), 1225 free (after 144 hours), 1226 bds (after 168 hours), 1226 free (after 168 hours), and 1227 bds (after 192 hours).

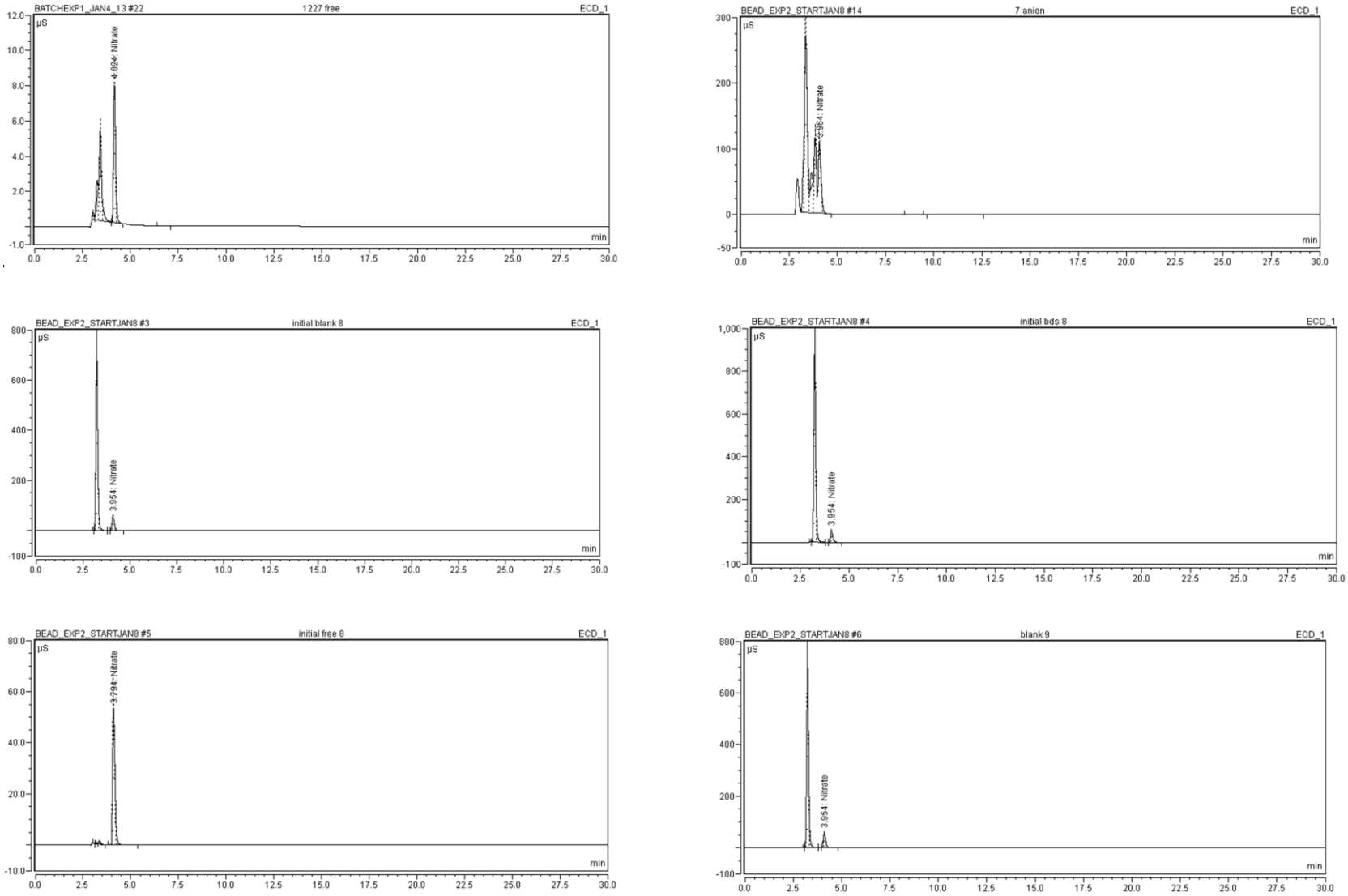


Figure C.4. Chromatograms of 1227 free (after 192 hours), 7A (after 0 hours), initial blank 8 (after 0 hours), initial bds 8 (after 0 hours), initial free 8 (after 0 hours), and blank 9 (after 24 hours).

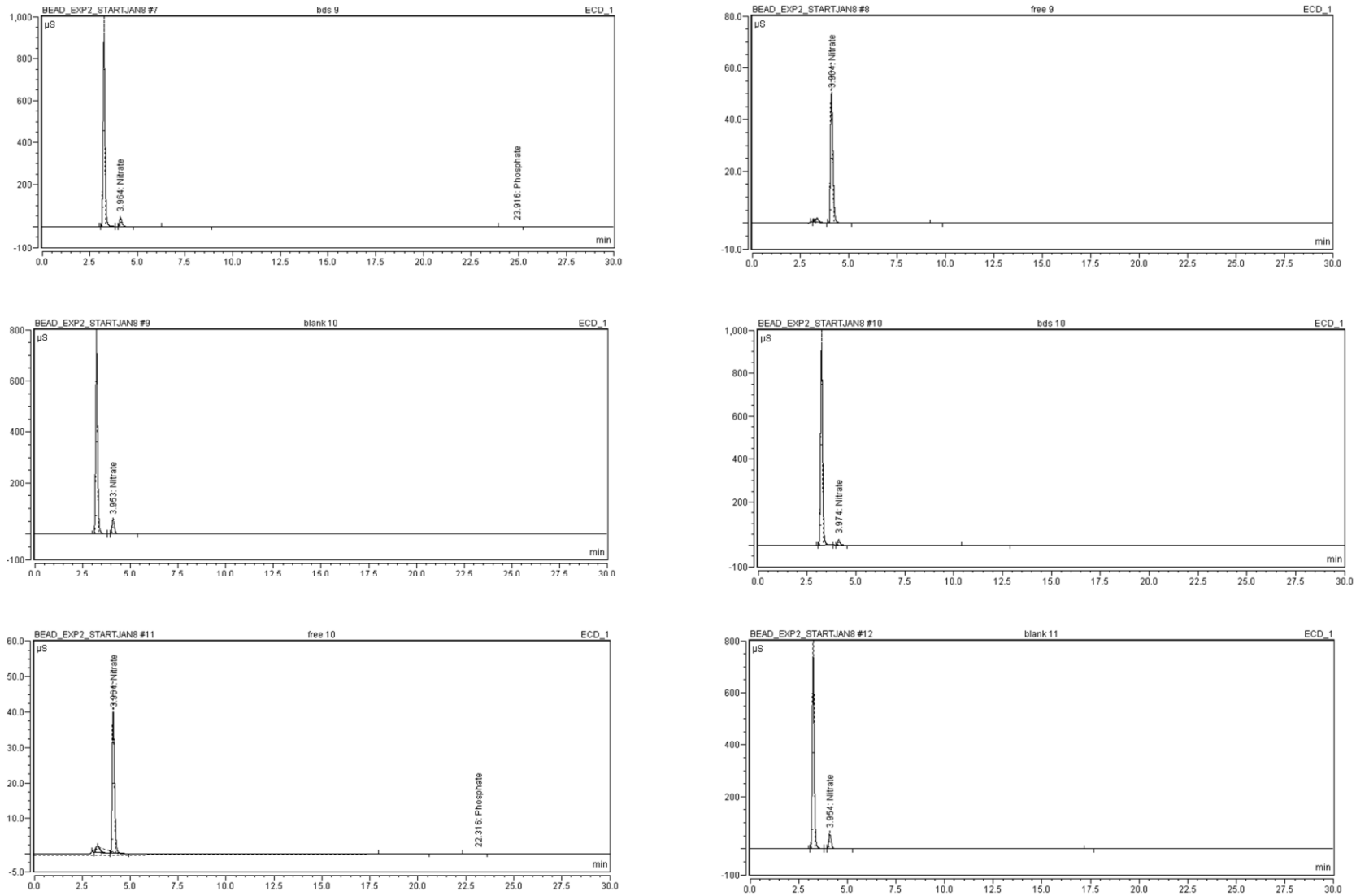


Figure C.5. Chromatograms of bds 9 (after 24 hours), free 9 (after 24 hours), blank 10 (after 48 hours), bds 10 (after 48 hours), free 10 (after 48 hours), and blank 11 (after 72 hours).

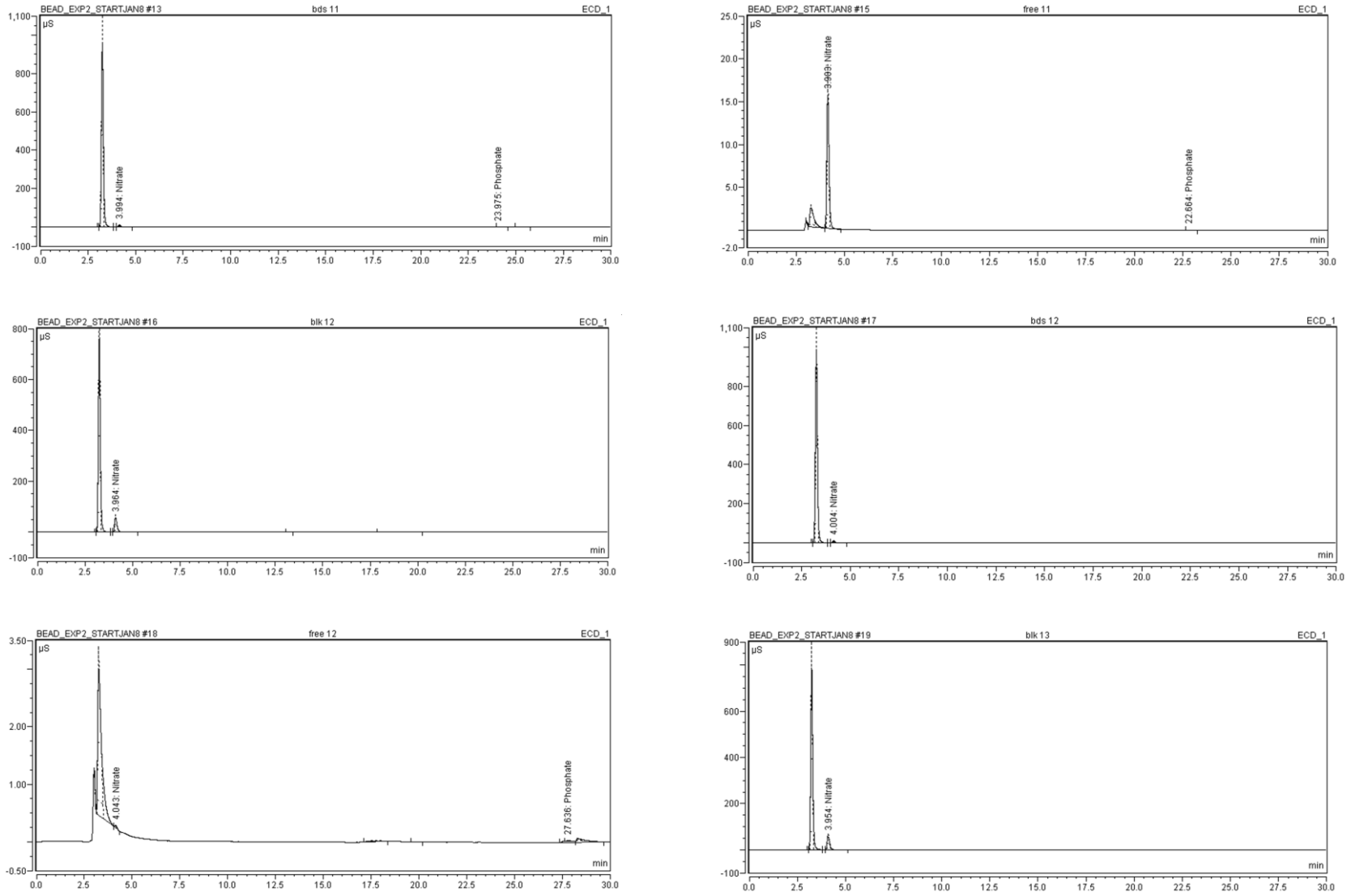


Figure C.6. Chromatograms of bds 11 (after 72 hours), free 11 (after 72 hours), blk 12 (after 96 hours), bds 12 (after 96 hours), free 12 (after 96 hours), and blk 13 (after 120 hours).

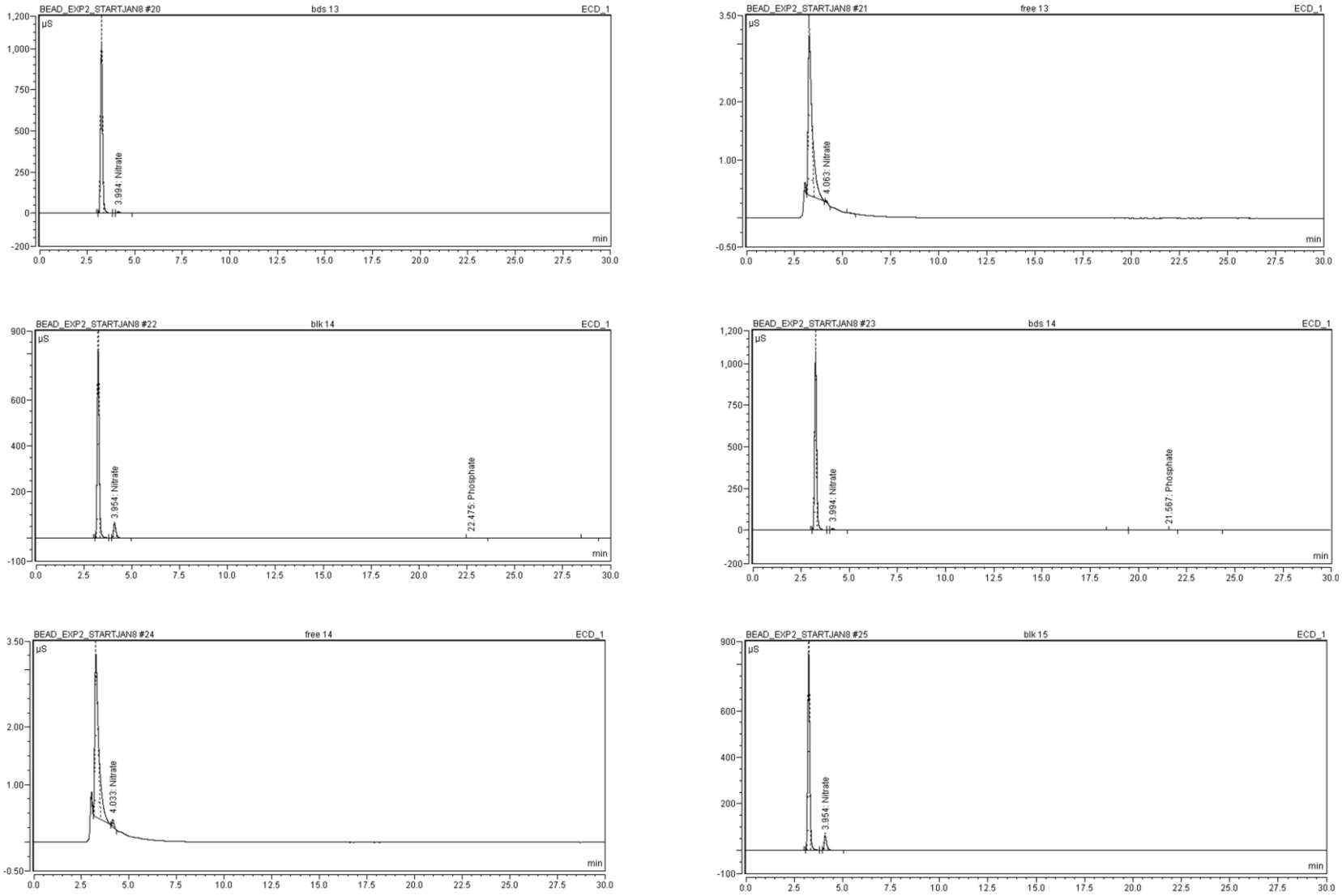


Figure C.7. Chromatograms of bds 13 (after 120 hours), free 13 (after 120 hours), blk 14 (after 144 hours), bds 14 (after 144 hours), free 14 (after 144 hours), and blk 15 (after 168 hours).

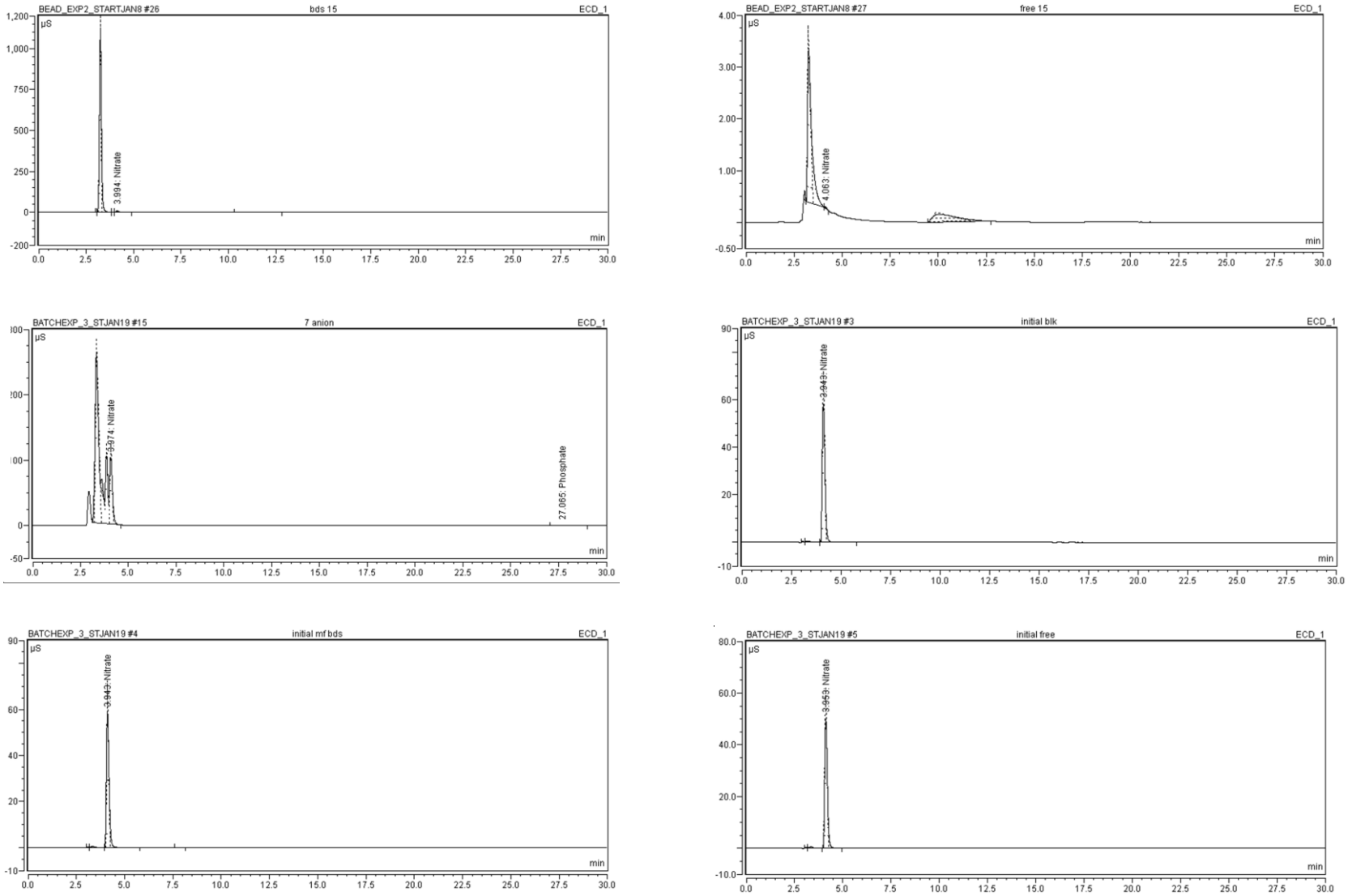


Figure C.8. Chromatograms of bds 15 (after 168 hours), free 15 (after 168 hours), 7A (after 0 hours), initial blk (after 0 hours), initial mf bds (0 hours), and initial free (after 0 hours).

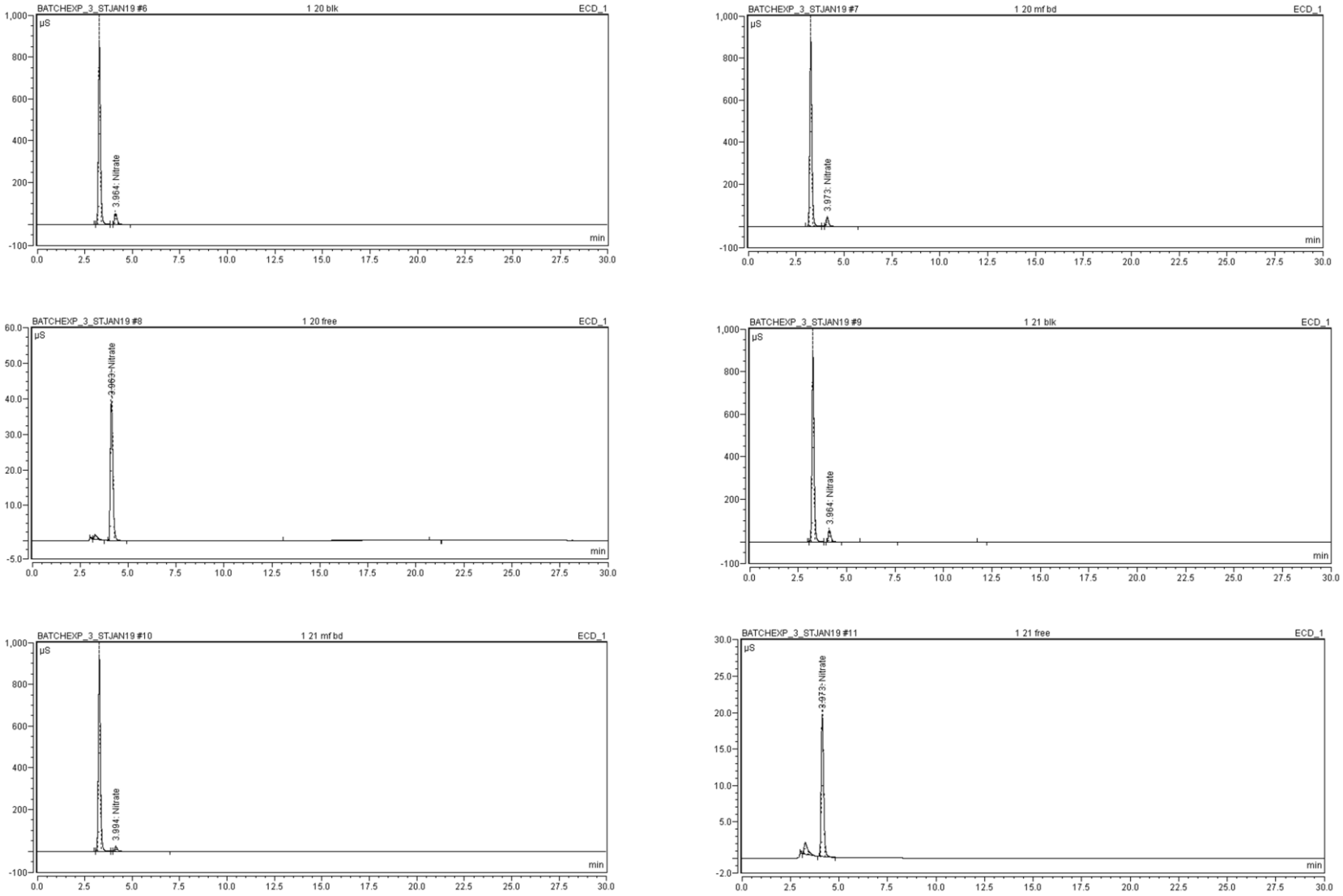


Figure C.9. Chromatograms of 1 20 blk (after 24 hours), 1 20 mf bd (after 24 hours), 1 20 free (after 24 hours), 1 21 blk (after 48 hours), 1 21 mf bd (after 48 hours), and 1 21 free (after 48 hours).

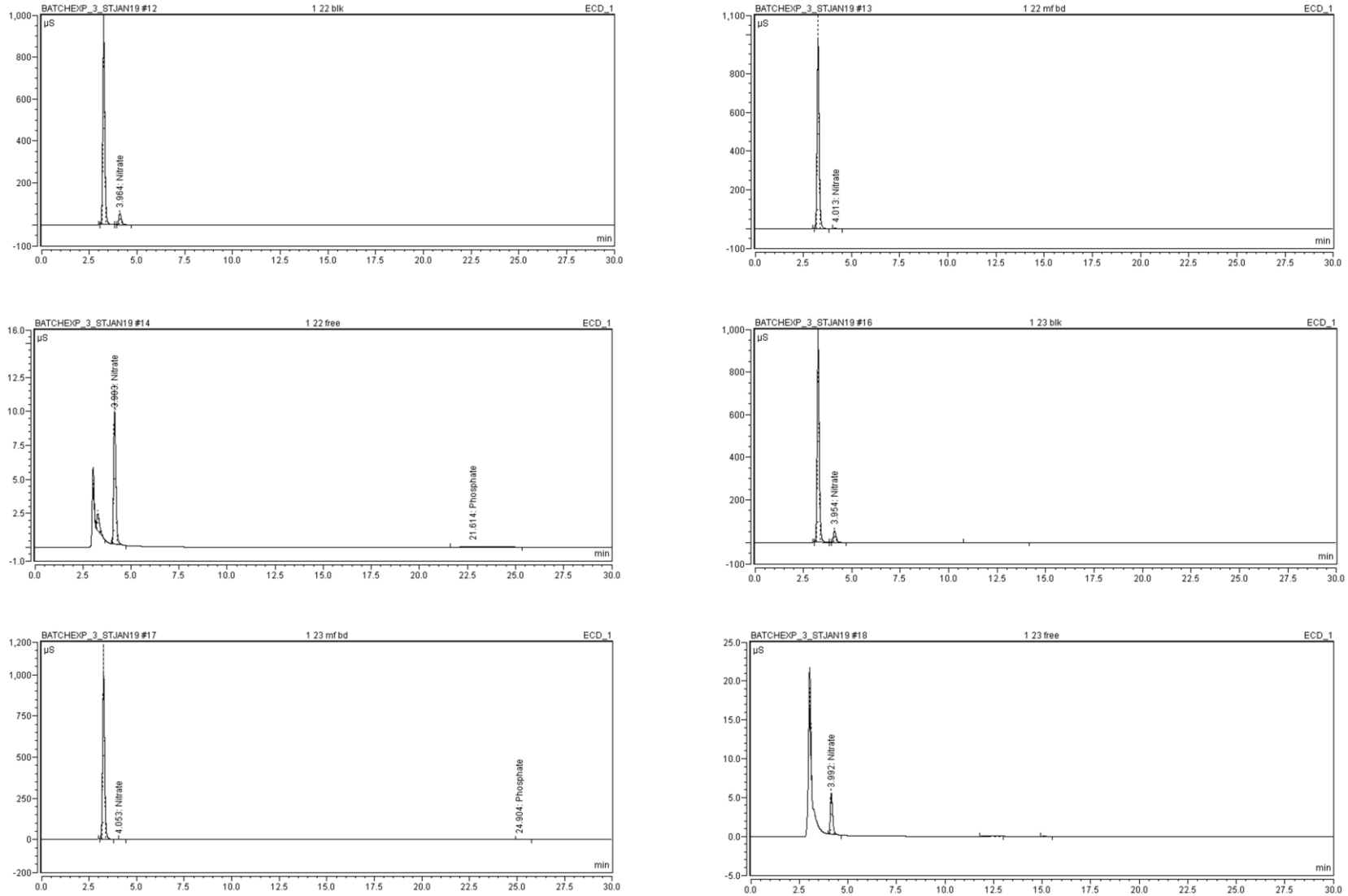


Figure C.10. Chromatograms of 1 22 blk (after 72 hours), 1 22 mf bd (after 72 hours), 1 22 free (after 72 hours), 1 22 blk (after 96 hours), 1 23 mf bd (after 96 hours), and 1 23 free (after 96 hours).

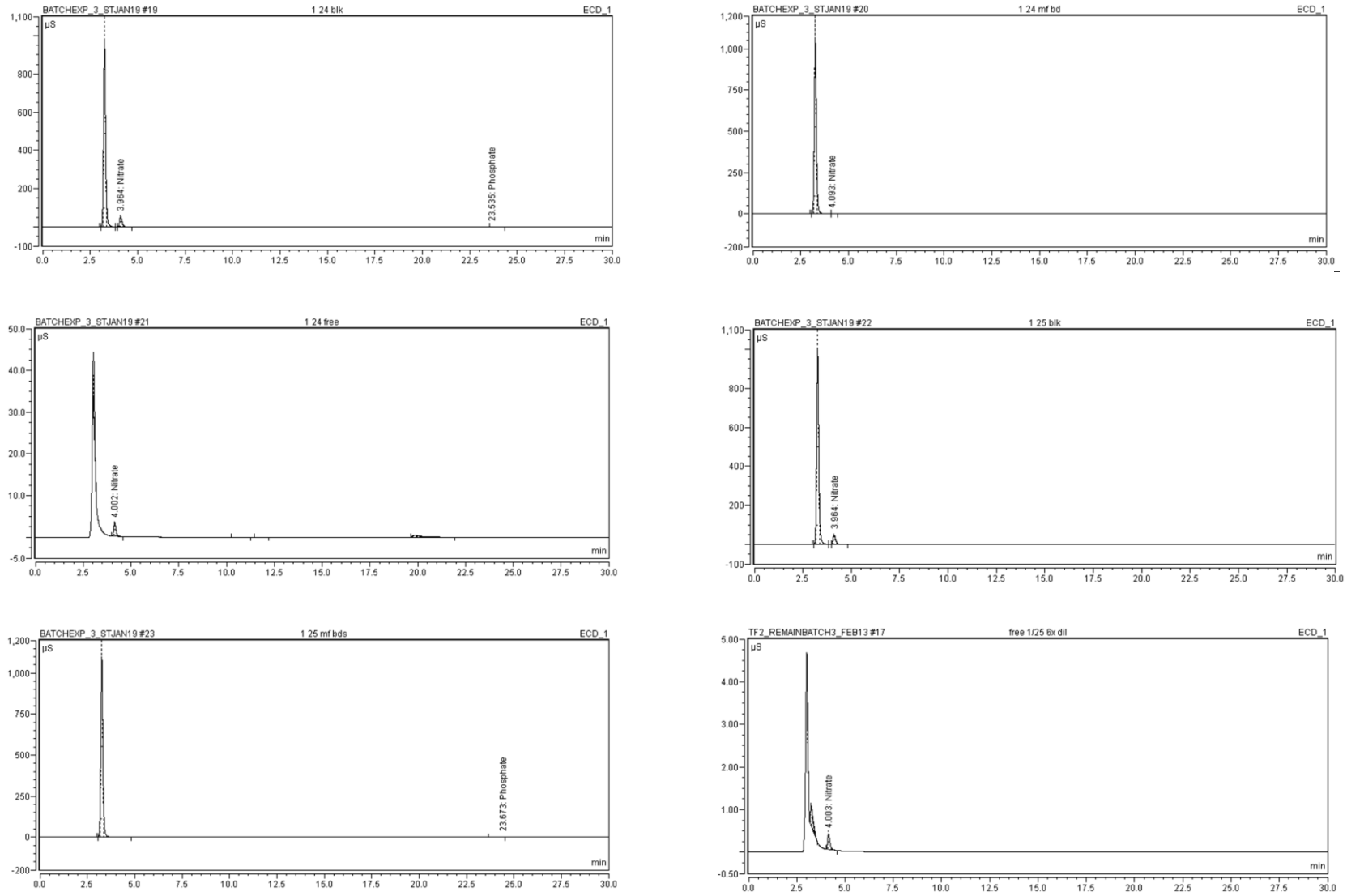


Figure C.11. Chromatograms of 1 24 blk (after 120 hours), 1 24 mf bd (after 120 hours), 1 24 free (after 120 hours), 1 25 blk (after 144 hours), 1 25 mf bd (after 144 hours), and free 1/25 6x dil (after 144 hours).

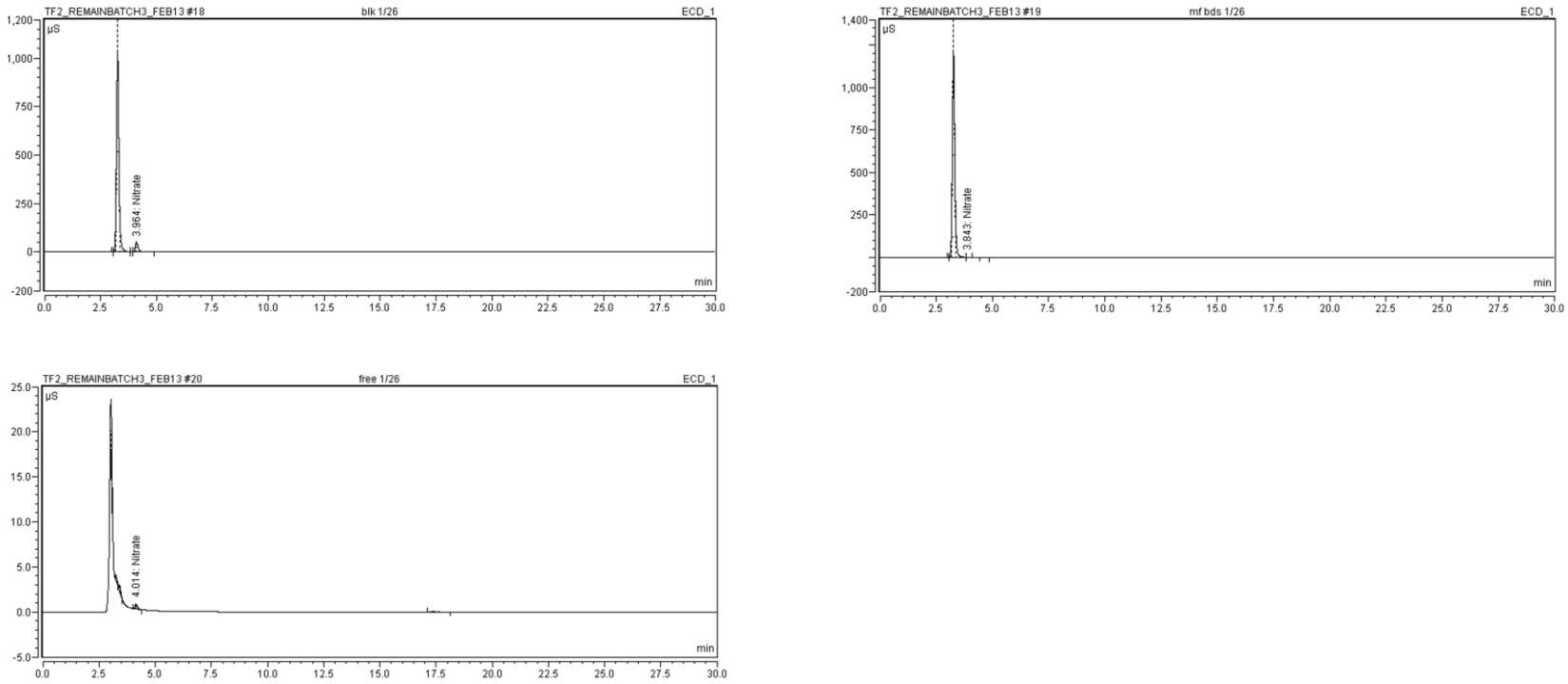


Figure C.12 Chromatograms of blk 1/26 (after 168 hours), mf bds 1/26 (after 168 hours), and free 1/26 (after 168 hours).

APPENDIX D: NO_3-N UTILIZATION IN A FLOW THROUGH FILTER DATA

TABLES AND CHROMATOGRAMS

Table D.1. Sample codes for NO_3-N utilization in a flow through filter samples.

Treatment Samples	Code
inoculated beads	bds
Reservoir Samples	
NO_3-N stock solution, initial concentration, inoculated beads	initial bds
Standard	
Dionex® Seven Anion Standard	7A

Table D.2. NO_3-N utilization in a flow through filter NO_3-N concentrations and corresponding chromatogram locations.

Chromatogram Label	Sample Code	Trial #	Hour	NO_3-N(mg/L)	Chromatogram Appendix #	Appendix page #
7 anion	7A	1	0	22.3	Figure D.1 (starting with 7 anion in the top left, moving left to right down the page, ending with 96 h 1 17 in the bottom right)	126
initial 1 13	initial bds	1	0	10.0		
24 h 1 14	bds	1	24	4.4		
48 h 1 15	bds	1	48	6.6		
72 h 1 16	bds	1	72	6.1		
96 h 1 17	bds	1	96	6.5		
120 h 1 18	bds	1	120	6.7	Figure D.2 (starting with 120 h 1 18 in the top left, moving left to right down the page, ending with tf2 48 h in the bottom right)	127
144 h 1 19	bds	1	144	6.5		
7 anion	7A	2	0	22.3		
initial tf 2	initial bds	2	0	9.0		
tf2 24 h	bds	2	24	1.5		
tf2 48 h	bds	2	48	1.3		
tf2 72 h	bds	2	72	2.3	Figure D.3 (starting with tf2 72 h in the top left, moving left to right down the page, ending with 7 anion in the bottom right)	128
tf2 96 h	bds	2	96	1.7		
tf2 120 h	bds	2	120	2.8		
tf2 144 h	bds	2	144	3.7		
tf2 164 h	bds	2	164	4.0		
7 anion	7A	3	0	22.3		
initial 2 7	initial bds	3	0	9.4	Figure D.4 (starting with initial 2 7 in the top left, moving left to right down the page, ending with 120 h in the bottom right)	129
24 h	bds	3	24	3.3		
48 h	bds	3	48	1.8		
72 h	bds	3	72	2.2		
96 h	bds	3	96	3.6		
120 h	bds	3	120	4.0		
144 h	bds	3	144	4.4	Figure D.5 (starting with 144 h in the top left, moving left to right down the page, ending with 185 h in the bottom left)	130
168 h	bds	3	168	0.9		
185 h	bds	3	185	3.1		

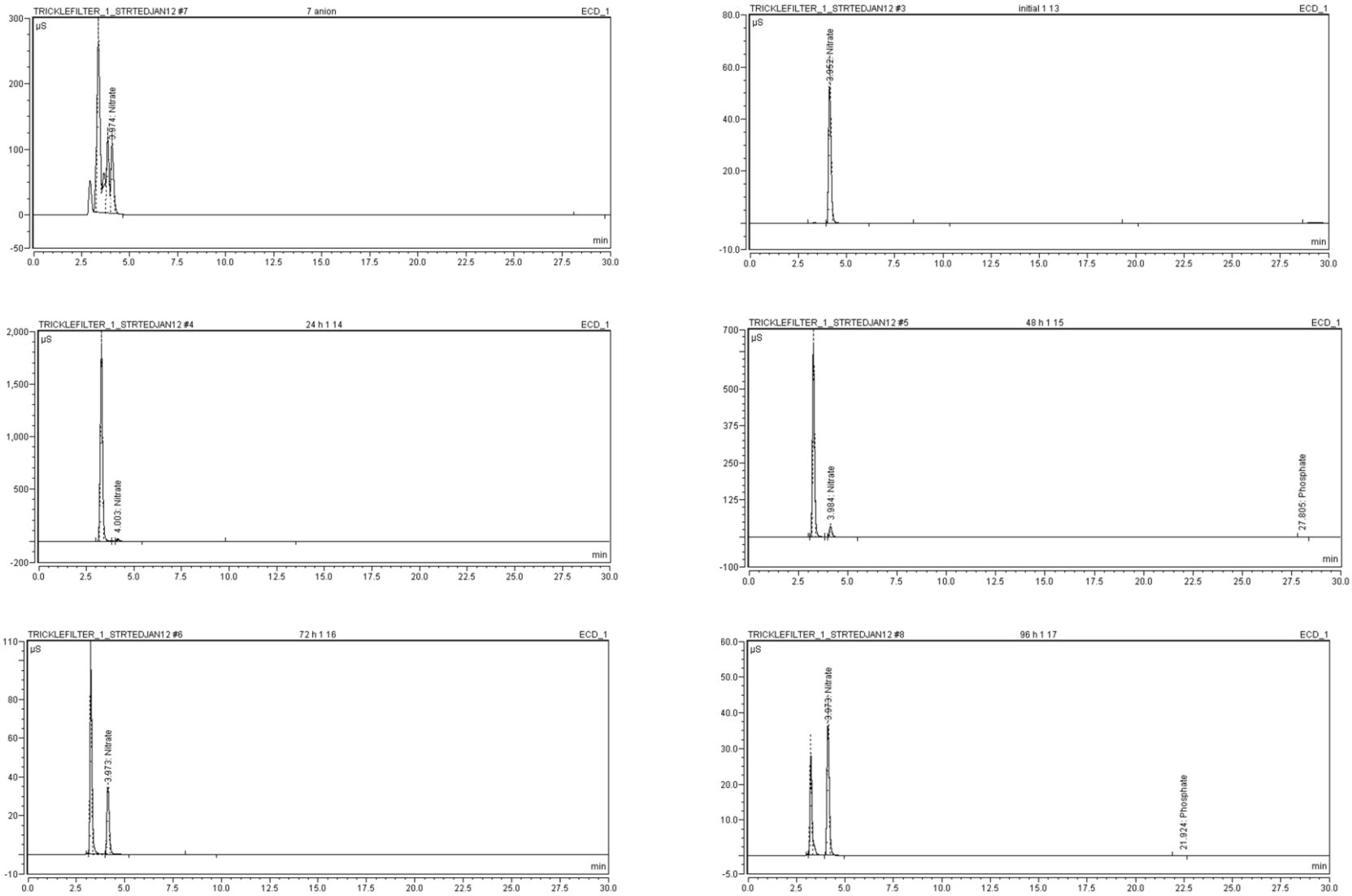


Figure D.1. Chromatograms of 7A (after 0 hours), initial 1 13 (after 0 hours), 24 h 1 14 (after 24 hours), 48 h 1 15 (after 48 hours), 72 h 1 16 (after 72 hours), 96 h 1 17 (after 96 hours).

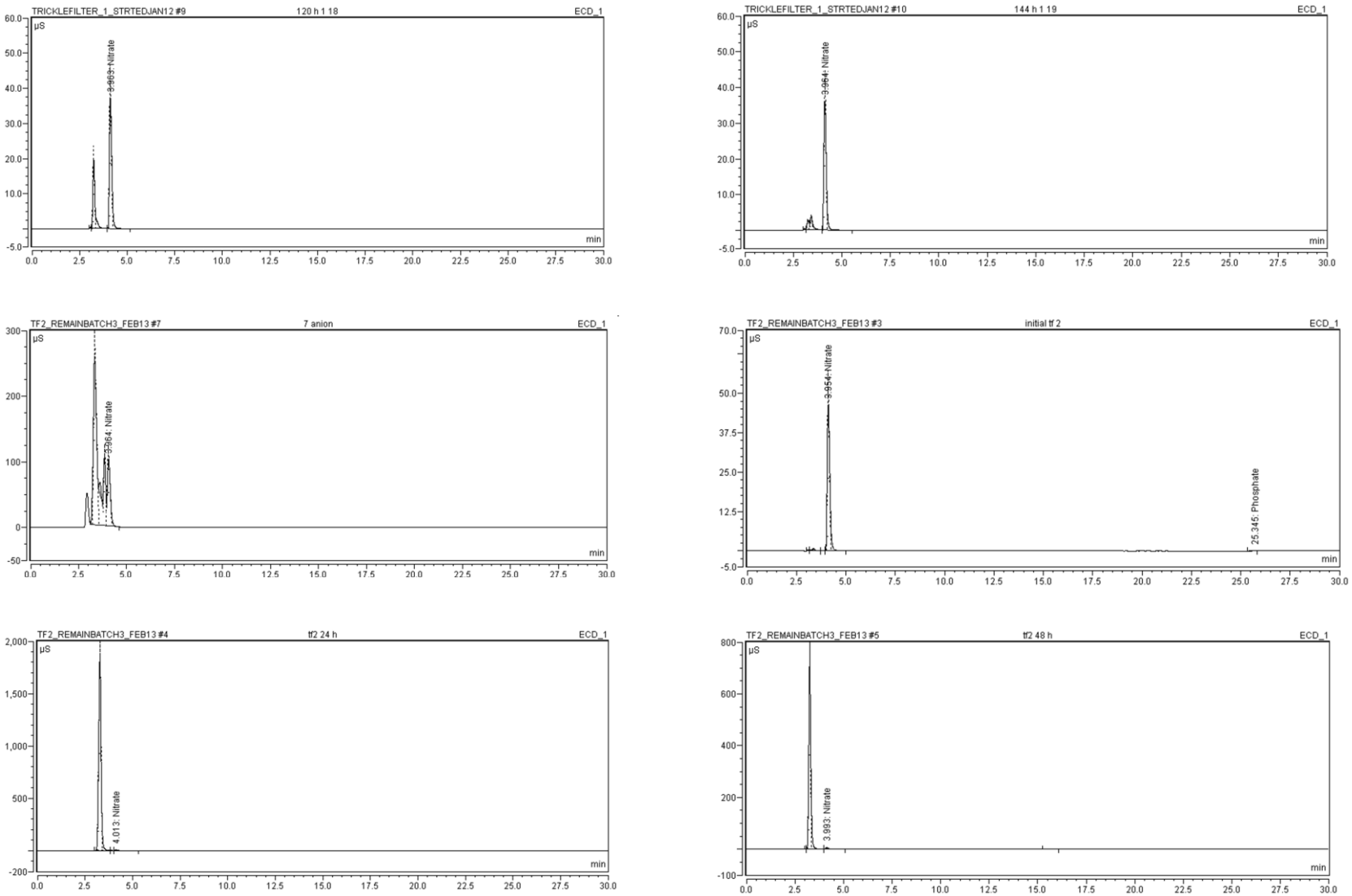


Figure D.2. Chromatograms of 120 h 1 18, 144 h 1 19, 7A (after 0 hours), initial tf 2 (after 0 hours), tf2 24 h, and tf2 48 h.

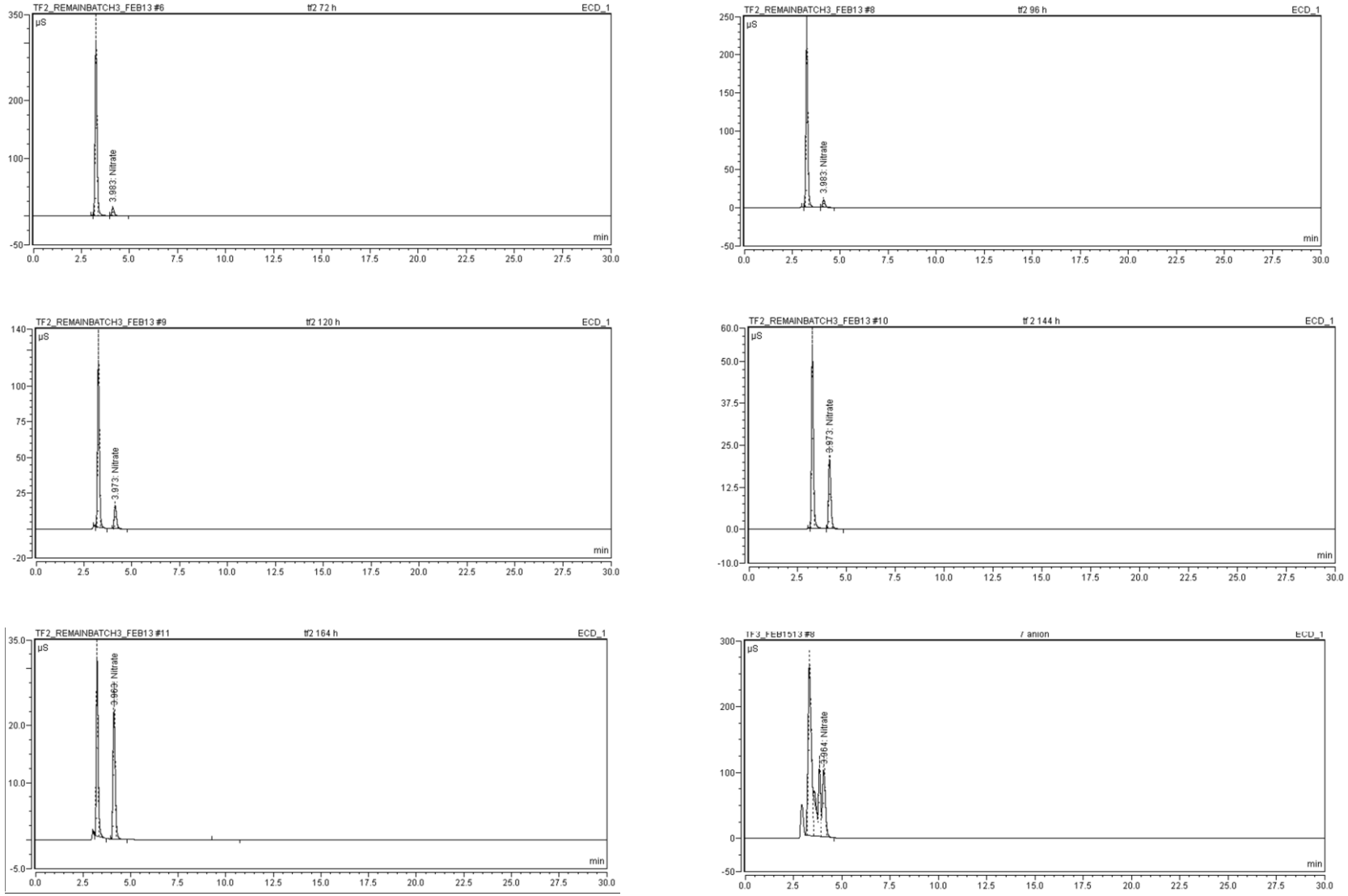


Figure D.3. Chromatograms of tf2 72 h, tf2 96 h, tf2 120 h, tf2 144 h, tf2 164 h, and 7A.

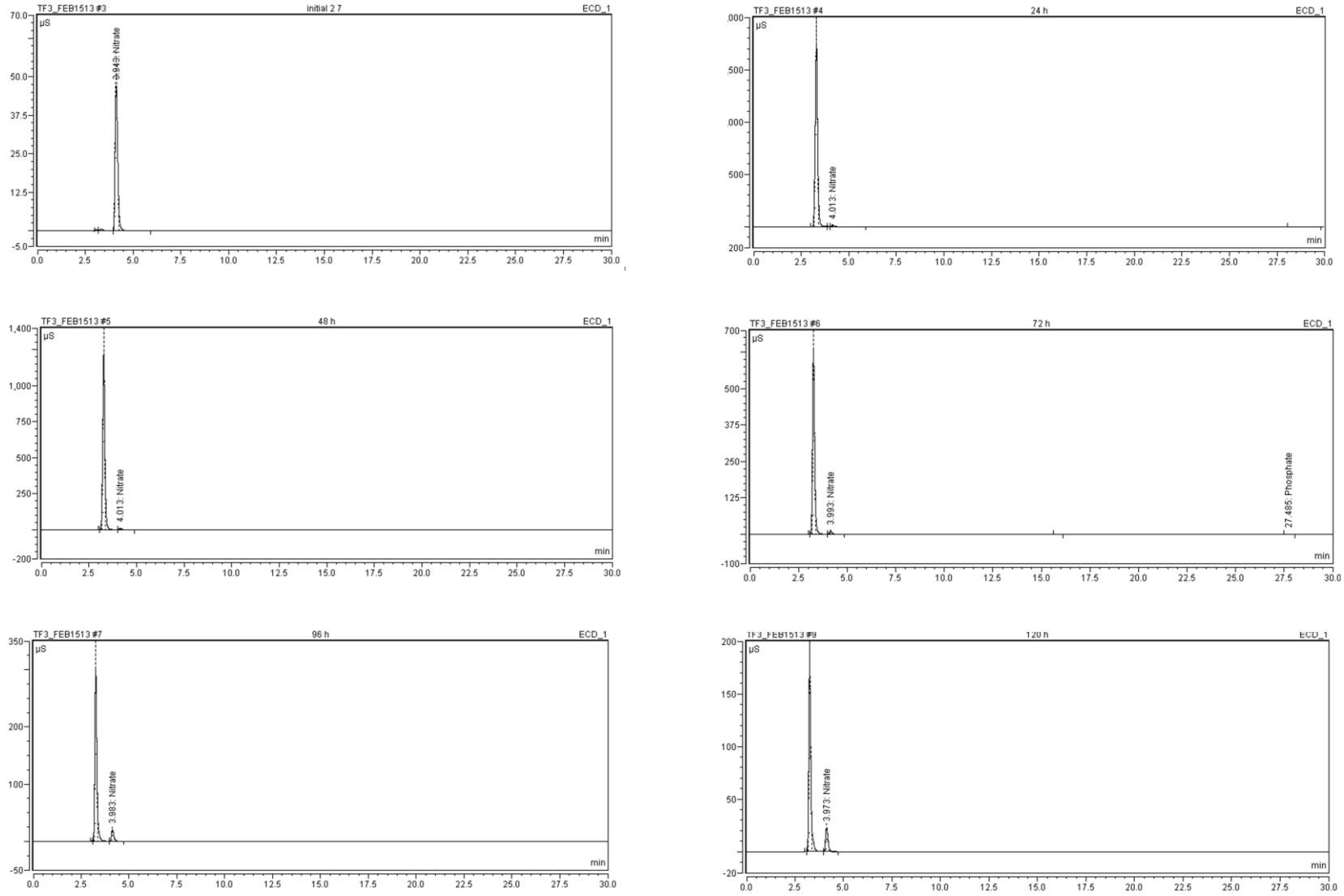
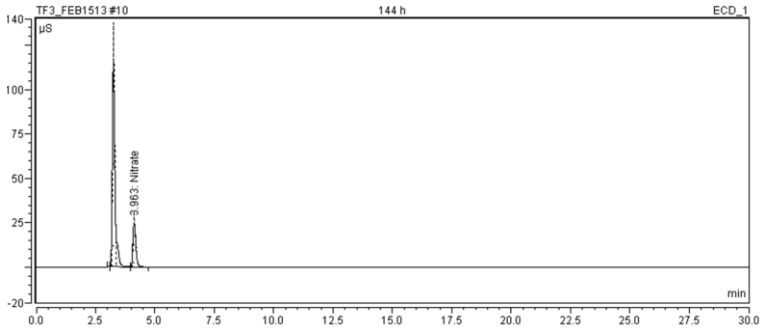
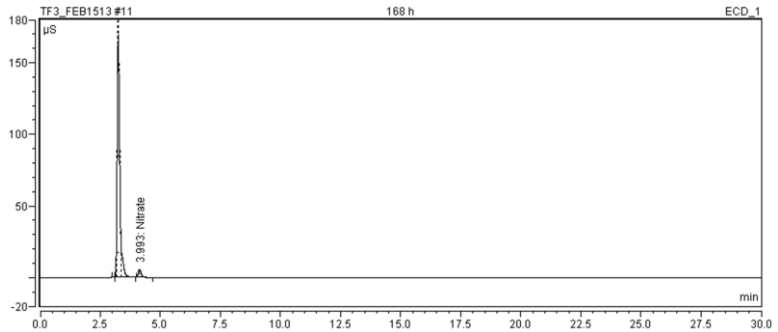


Figure D.4. Chromatograms of initial 2 7 (after 0 hours), 24 h, 48 h, 72 h, 96 h, and 120 h.





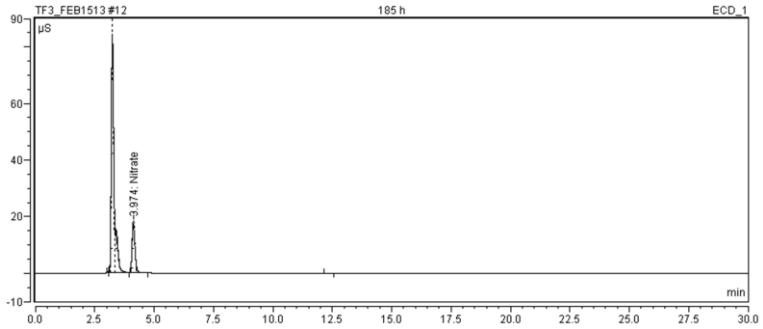


Figure D.5. Chromatograms of 144 h, 168 h, and 185 h.